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# Adaptation of muscle size and myofascial force transmission: a review and some new experimental results

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**This paper considers the literature and some new experimental results important for adaptation of muscle fiber cross-sectional area and serial sarcomere number. Two major points emerge: (1) general rules for the regulation of adaptation (for *in vivo* immobilization, low gravity conditions, synergist ablation, tenotomy and retinaculum trans-section experiments) cannot be derived. As a consequence, paradoxes are reported in the literature. Some paradoxes are resolved by considering the interaction between different levels of organization (e.g. muscle geometrical effects), but others cannot. (2) An inventory of signal transduction pathways affecting rates of muscle protein synthesis and/or degradation reveals controversy concerning the pathways and their relative contributions.**

**A major explanation for the above is not only the inherently limited control of the experimental conditions *in vivo*, but also of *in situ* experiments.**

**Culturing of mature single *Xenopus* muscle fibers at high and low lengths (allowing longitudinal study of adaptation for periods up to 3 months) did not yield major changes in the fiber cross-sectional area or the serial sarcomere number. This is very different from substantial effects (within days) of immobilization *in vivo*. It is concluded that overall strain does not uniquely regulate muscle fiber size.**

**Force transmission, via pathways other than the myotendinous junctions, may contribute to the discrepancies reported: because of substantial serial heterogeneity of sarcomere lengths within muscle fibers creating local variations in the mechanical stimuli for adaptation. For the single muscle fiber, mechanical signalling is quite different from the *in vivo* or *in vitro* condition. Removal of tensile and shear effects of neighboring tissues (even of antagonistic muscle) modifies or removes mechanical stimuli for adaptation.**

**It is concluded that the study of adaptation of muscle size requires an integrative approach taking into account fundamental mechanisms of adaptation, as well as effects of higher levels of organization. More attention should be paid to adaptation of connective tissues within and surrounding the muscle and their effects on muscular properties.**

## The need for understanding muscular adaptation

Major determinants of the capability of movement induced by a muscle activity are (1) the maximum (optimal) force that can be exerted by an active muscle and (2) the length at which optimal force is exerted (muscle optimum length), as well as (3) its length range of active force exertion. These factors can readily be quantified in experimental research on experimental animals, but are far more difficult to assess in humans.

Optimal muscle force is largely determined by the physiological cross-sectional area of the muscle ($A_f$, usually defined at optimum length). The ratio of muscle volume to (mean) muscle fiber optimum length (or alternatively mean fiber length at muscle optimum length) yields a fairly good estimate for $A_f$. It should be noted that this is also true for very pennate muscle in which the estimate has been very often confounded by erroneously introducing angular factors into the calculation (e.g. Fukunaga et al., 1996). Another important factor is the measurement of fiber or fascicle lengths at a standard muscle length (e.g. optimum length or another standard).

The muscular length range of active force exertion is determined primarily, but not exclusively, by the number of sarcomeres arranged in series within the muscle fibers (for a review on additional contributing factors see Huijing, 2000). Unfortunately, this factor cannot readily be estimated for *in vivo* muscles.

These prime parameters $A_f$ and serial sarcomere number are adjusted so finely to functional demands in daily life, that healthy people can use their muscles without much attention to the tasks to be executed. This means that as the body grows the fine-tuning to functional tasks remains. A possible exception may

be the growth spurt during initial phases of puberty. However, in many pathological cases, muscular properties may limit such easy use of muscle. For example, a loss of muscle mass may occur in pathologies such as cerebral palsy (Sage, 1992; Young, 1994), spinal cord injury (Thomas et al., 1997; Castro et al., 1999), chronic obstructive pulmonary disease (Gosker et al., 2000) as well as cardiac cachexia (Mancini et al., 1992; Gosker et al., 2000; Schulze et al., 2002). In such cases, muscle fibers are not adapted optimally (regarding $A_f$ and/or serial sarcomere number) to the daily tasks, causing limited mobility in those patients.

The development of effective therapy requires a fundamental knowledge of the mechanisms affecting hypertrophy/atrophy and adaptation of the number of sarcomeres in series (below further referred to as serial sarcomere number). Also for sports sciences such knowledge may be important to develop improved training methods.

This review will integrate, on the one hand, results regarding adaptation of the prime parameters $A_f$ and serial sarcomere number from experiments performed *in vivo*, *in situ* as well as *in vitro*, with those of mathematical modelling of muscle (bioengineering) on the other hand. Such a study involves very different levels of organization, at the level of (a) the organism, (b) the limb, (c) the muscle group, (d) the organ (i.e. muscle) and (e) the cell (i.e. muscle fiber).

Therefore, for the study of muscle properties an integrative approach with respect to the level of organization has recently been argued to be necessary (Huijing, 2003), because of effects of myofascial force transmission. This type of force transmission will also be considered below.

## Classical animal experimentation and some human *in vivo* experiments

Our, rather limited, insights into mechanisms of muscular adaptation are based predominantly on these types of experiments. Several relatively simple to impose experimental conditions have been used to study *in vivo* effects on $A_f$ and serial number of sarcomeres, as well as functional consequences of any such adaptation. Note that in such animal experiments the functional assessments were always made on fully dissected muscle active *in situ*.

Researchers in this field are usually faced immediately with a difficult decision: To what should the results of an experimental muscle be compared? There are usually two options, each with its own advantages and disadvantages. (1) Comparison with the muscle within contra-lateral leg of the animal. An obvious advantage is that inter-individual variance does not affect the results. In this case the sometimes-implicit assumption is made that the contra-lateral muscle serving as control is not affected by the intervention. It is clear that such an assumption may be false in many cases: it is unlikely that the use of the contralateral limb will be unchanged, if very substantial changes are imposed on the experimental limb of an animal or human. In some cases it is clear that changes in some parameters of the contra-lateral muscle may be higher than in the experimental muscle (Heslinga & Huijing, 1993). (2) Comparison with muscles of other individuals not undergoing the intervention avoids comparisons between muscles that both may have adapted, but introduces inter-individual variance into the results.

Unfortunately, in this type of work, longitudinal studies studying effects and mechanisms of adaptation are usually not possible.

### Natural growth

In young animals that are growing in body size, the growing bones will stretch the muscles and keep them dynamically active at relatively higher lengths and possibly enhanced length ranges because of increased moment arms. It is obvious that such conditions constitute a signal for adaptation of serial sarcomere number as well as $A_f$. Generally, in healthy animals for both variables sizable increases have been reported accompanied by increase in optimum length (Crawford, 1954; Goldspink, 1964, 1968; Williams & Goldspink, 1971; Tardieu et al., 1977; de Koning et al., 1987; Heslinga & Huijing, 1990; Heslinga et al., 1995).

### Immobilization of joints

#### *Muscle kept at low length*

In this experiment, sustained maximum ankle plantar flexion in a plaster cast immobilized cat or rodent soleus muscle at low length was maintained (Tabary et al., 1972; Goldspink et al., 1974; Williams & Goldspink, 1978; Spector et al., 1982; Heslinga & Huijing, 1993). Consistently, such experiments yielded reductions in the number of sarcomeres in series of m. soleus ($-25\%$ to $-40\%$) after several weeks of immobilization. Findings consistent with this were also reported for the diaphragm for emphysematous rats (Shrager et al., 2002).

Also in m. soleus, $A_f$ decreased by 35–40% after low length immobilization (Spector et al., 1982; Heslinga et al., 1995). As a consequence optimal force was reduced, and this force was attained near the length of immobilization, i.e. at a considerable shortening of the muscle (Williams & Goldspink, 1978).

Such results have led to the classical concept that the actual experimental condition of the muscle

determines the processes of adaptation (Williams & Goldspink, 1978).

Intact innervation does not seem to be a major factor in this type of adaptation, as in denervated muscles immobilized at low length the serial sarcomere number was reduced equally as within muscles with intact innervation. The process of adaptation occurred only slower (Goldspink et al., 1974; Hayat et al., 1978).

However, contrasting findings are reported also for adaptation of serial sarcomere number, particularly for highly pennate muscle. Tardieu et al. (1974) found no change in serial sarcomere number for cat tibialis anterior muscle after immobilization at low length. In contrast, Tabary et al. (1972, cat soleus muscle) as well as Heslinga and Huijing (1993, rat soleus) found substantially decreased serial sarcomeres numbers ($-20\%$ to $-40\%$). However, for medial gastrocnemius muscle of the same rats (Heslinga & Huijing, 1993) immobilized at similar normalized fiber lengths they found no decrease in serial sarcomere number, but did find substantial decreases in optimum muscle length as well as significant atrophy.

Immobilization at low lengths, interrupted by short periods during which the muscle was kept at high lengths, also contributed to recognizing the very high adaptive signal implicit in placing the muscle at high lengths. Gomes et al. (2004) imposed maximally high lengths (presumably through maximal dorsal flexion of the ankle) only once a week for 40 min, on rat soleus muscle immobilized in shortened position. This short high length exposure provided significant protection against muscle fiber atrophy. In contrast, it was not sufficient to prevent the reduction of muscle weight and of serial sarcomere number. Earlier Williams (1990) had shown that periods at high lengths as short as 1/2 an hour daily were sufficient, not only to prevent loss of sarcomeres, but actually to cause an increase in serial sarcomere number.

#### *Muscle kept at high length*

The findings regarding the powerful effects of temporarily keeping immobilized muscle at high lengths are consistent with the general anabolic effects of stretching a muscle to high lengths reported by Goldspink (1977). Therefore, it is not surprising that permanent immobilization at high lengths yielded increases in serial sarcomere number and at least the prevention of major atrophy or even increased muscle fiber diameters (Crawford, 1954; Tabary et al., 1972; Tardieu et al., 1974; Williams & Goldspink, 1978). Consistent findings were also reported for the diaphragm for emphysematous rats after lung volume reduction surgery (Shrager et al., 2002).

An important finding of those studies on muscles of the lower limb was again that the new optimum length is found near the length of immobilization.

For muscles immobilized at high lengths (i.e. over optimum length), the increase in the serial sarcomere number was not affected by muscular activity (Williams & Goldspink, 1978). These findings indicate that activity is not necessary for adaptation of the serial sarcomere number.

### Low gravity conditions and limb suspension and bed rest

Human space travel has led to a highly increased interest in effects of micro-gravity on skeletal muscle. On earth, this has also led to enhanced interest in the effects of limb or body suspension, presumably being a valid model for low gravity conditions. A plethora of literature can be found indicating that skeletal muscles are vulnerable to marked atrophy under micro-gravity (Nikawa et al., 2004). Sometimes, such atrophy is reported in terms of changes in muscle volume (Akima et al., 2000) and sometimes as decreases in physiological cross-sectional area of muscle (Miyamoto et al., 1998; Wimalawansa et al., 1999) or muscle fibers (Roy et al., 1999a). Even short periods of unweighting because of micro-gravity or limb suspension result in decreases in the cytoplasmic volume-to-myonucleus ratio (Kasper & Xun, 1996).

Somewhat surprisingly, we have not been able to find reports regarding effects of micro-gravity or unweighting on variables of length–force characteristics or serial sarcomere number.

It is clear that, also in this area of research, effects of actual length of the muscle or tendon complex are often disregarded. How important such effects may be, is evident from the finding reported by Goldspink et al. (1986) that the much smaller atrophy of extensor muscles in suspended limbs represents an underestimate of the true atrophic effect because of high length-related protein synthesis enhancement in those muscles. It is even conceivable that at least some of the differential atrophy effects reported for muscles of different fiber type compositions may be related to such length effects.

### Tenotomy, myotomy or denervation of synergistic muscles: overloading agonistic muscle

A large body of literature may also be found on what is called compensatory hypertrophy after tenotomy, or more often ablation, of synergist muscles. It should be noted that synergist tenotomy does not always have the expected hypertrophy effect (Ohira, 1989). Regarding many effects, related to compensatory hypertrophy, the literature is contradictory. This is particularly true for the question of occurrence of hyperplasia. A meta-analysis of 17 studies has been

suggested to show significant increases in muscle fiber number (hyperplasia) (Kelley, 1996). However, previously, severe criticism had been directed (e.g. Taylor & Wilkinson, 1986) to, at least, some of the methods used to estimate muscle fiber number experiments. This criticism led to the conclusion that hyperplasia has not yet been substantiated.

On the other hand, after compensatory hypertrophy many small diameter fibers were seen in muscles undergoing such hypertrophy. These fibers appear to be new fibers arising from satellite cells. They were not seen after irradiation, which prevents hypertrophy by impairing activation, proliferation and/or differentiation of satellite cells (Phelan & Gonyea, 1997). Yet, an increased number of branched muscle fibers (Tamaki et al., 1996) may very well explain findings interpreted as hyperplasia. In such a case, the branching of muscle fibers should be interpreted more as incomplete fusion during a process of regeneration after damage rather than splitting of muscle fibers.

A usually very sizable increase in muscle mass and hypertrophy of muscle fibers is a very general finding (see most references in this paragraph). An interesting observation may be the following contrast between two types of results found sometimes: on the one hand, we have muscle fiber type conversion to slow types (i.e. an increase in percentage of type I muscle fibers; Degens et al., 1995), as well as an increasing slower myosin heavy chain (MHC) isoforms and a concomitant decrease in the faster MHC isoforms (Stone et al., 1996). On the other hand is the finding that isometric twitch time to peak tension remains unaltered. This could lead to the hypothesis that the slower intrinsic velocity of contraction is compensated by an increased number of serial sarcomeres. Freeman and Luff (1982) reported such an increase in the number of serial sarcomeres.

The results even of ablation experiments have been interpreted almost exclusively in simple terms of effects of overload of the remaining agonistic muscles. However, apart from myofascial effects to be discussed below, it is already clear that the surgical intervention itself creates many signals for adaptation: activated fibroblasts displaying a vesicular nucleus with prominent nucleoli and an outstanding increase in cytomembranes, particularly the rough endoplasmic reticulum, and the Golgi complex were reported for both sham-operated and experimental animals (Zamora & Marini, 1988). Damage, rather than an increase in muscle activity, may play a more significant role in at least the early activation of satellite cells during compensatory hypertrophy (Snow, 1990).

Such conclusions are also supported by the similar overall effects on muscle seen after injection of damaging muscle compounds (Rosenblatt & Woods, 1992).

In any case, compensatory hypertrophy should also be regarded with myofascial effects in mind.

### Retinaculum release: changing muscle–tendon complex length and moment arm

It has been hypothesized that the serial sarcomere number is determined by the magnitude of excursion performed by the muscle (e.g. Herring et al., 1984; Burkholder & Lieber, 1998; Koh & Herzog, 1998). For a start, this seems to be quite a reasonable hypothesis that may be derived from the fact that during natural growth with increased body size, both moment arms and serial sarcomere numbers increase substantially. Retinaculotomy is supposed to increase the moment arm (i.e. the distance between the muscle line of pull and the axis of joint rotation) and therefore leads to enhanced excursion of the muscle for identical joint ranges. It also leads to a changed joint angle muscle length relation: for a given joint angle the muscle–tendon complex will be shorter. The presence of two opposing signals (short muscle, increased length range) made it an apparently elegant experiment to identify the most important parameter for adaptation of muscle size. However, experiments designed to do this, yielded opposite conclusions: Burkholder and Lieber (1998, adult mouse anterior tibial muscle) found a reduction of serial sarcomere number, Koh and Herzog (1998, young rabbits) found an increase. Such contrasting findings regarding the adaptation of the serial sarcomere number indicate that either species differences may be very important or the imposed *in vivo* conditions may not have been similar.

### Explaining some paradoxes: adaptation results are dependent on muscle and tendon architecture as well as on age

#### *Immobilization of young growing animals*

Immobilization of muscle of young animals *at high length* yielded similar results to immobilization of adult animals *at low length* (Tardieu et al., 1977). Tardieu et al. (1977) also reported how to interpret this initially baffling result: the length of tendon of the young muscles immobilized at high length increased very much changing the experimental conditions of the muscle belly and of the fibers from high- to low-length conditions during the experiment.

#### *Effects of hyper or atrophy in pennate muscle*

In parallel-fibered muscle or pennate muscle of a low degree (i.e. muscles with the line of pulls of muscle and of its fibers almost parallel) the effects of adapting $A_f$ and serial number of sarcomeres are independent: for example, decreasing the serial sarcomere

number shortens the muscle and atrophy decreases muscle thickness. Each one of these factors does not affect the other. In contrast, because of geometrical effects, changes of the diameter of muscle fibers of very pennate muscle will also affect muscle length. One of the first authors to consider such consequences of muscle geometry in the analysis of adaptation effects was Swatland (1980).

Another paradox: even within the same young adult rat, the soleus muscle adapts serial sarcomere number during growth and immobilization, but the overlying gastrocnemius muscle does not. Also, this paradoxical finding was resolved by taking into account the geometric effects of very pennate gastrocnemius muscle (Heslinga & Huijing, 1993; Heslinga et al., 1995). This means that during the period of immobilization, atrophy released the muscle from the low fiber length-related signal to remove sarcomeres in series. This happens as follows: the muscle is kept in the maximally *in vivo* shortened position with short fiber and sarcomere lengths. In an unrestrained muscle, progressive atrophy would lead to further muscle belly shortening without changing fiber and sarcomere lengths, exclusively because of the geometric effect. If the muscle length is the restrained variable, the muscle belly cannot *shorten because of atrophy*, but can exclusively accommodate the geometric effect by *lengthening* its fibers and sarcomeres (Fig. 1). If this effect of atrophy is large enough to bring the fibers near their optimum length, we should expect the net long-term effects of immobilization at optimum length. Such adaptation does not include adaptation of serial sarcomere number.

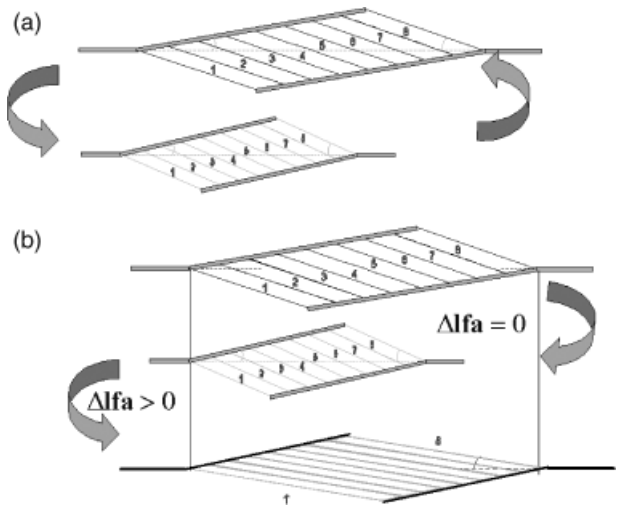


*Fig. 1.* Schematic representation of effects of atrophy and hypertrophy and immobilization on lengths of muscles and fibers of pennate muscle. (a) Unrestrained condition. Note that pennate muscle will drastically change its length with changes in fiber diameter because of atrophy or (hyper-) trophy. (b) Immobilized condition. Atrophy would lead to a shortened length in pennate muscle. Because of the restraint of immobilization the muscle can only keep its length by lengthening muscle fibers. The arrow indicates immobilized length.

Similar effects also explain the paradox of opposing results described above for retinaculum release experiments of Koh and Herzog (1998) and Burkholder and Lieber (1998). Retinaculum release has two acute effects: (1) shortening of the muscle and muscle fibers, because of a more direct path of the tendon from insertion to the muscle belly and (2) increasing the moment arm of the muscle at the joint, so that increased muscular length ranges are necessary to move the joint through an identical angle range as prior to the retinacular release.

In their argumentation, Koh and Herzog failed to take into account additional effects of muscle atrophy, which they reported for the experimental leg with respect to the control muscle. Following the argumentation presented above, we draw the following conclusion: because of major atrophy, the muscle fibers of rather pennate tibialis anticus (TA) muscle were active at higher lengths than before the atrophy. Therefore, one should expect adaptation, i.e. an increased serial sarcomere number, based on effects of high fiber lengths, instead of causally relating increased serial sarcomere number and the enlarged muscular excursion. Results for the adult mouse muscle with resected retinaculae (Burkholder & Lieber, 1998) confirm such reasoning: in this case the physiological cross-sectional area ($A_f$) was not changed, and therefore any confounding change in the relation of muscle length and muscle fiber length is not likely.

Therefore, the finding that the enlarged excursion of these muscles was accompanied by a reduction in the serial sarcomere number seems to support the notion that excursion per se is not a most important regulator of the serial sarcomere number. In agreement with previous literature on muscular adaptation, the specific conditions of the experiment of Burkholder and Lieber, being the lower length of muscle fibers, because of retinacular release must have been the determining factor for adaptation of serial sarcomere number.

A major lesson to be drawn from these paradoxes is that the conditions of the muscle fibers and not of the muscle–tendon complex or muscle seem to determine the adaptation effect. This means that always effects of serial elasticity, effects of pennation, etc. and changes thereof have to be taken into account before adaptation mechanisms can be adequately studied.

## Molecular mechanisms of adaptation of muscle size

Despite the controversies shown by the classical experiments, such experiments have taught us that

at least some physiological conditions are able to trigger adaptation of muscle size. However, this is only a limited aspect of the unravelling of the mechanisms underlying the regulation of muscle size. Advances in molecular biology have facilitated the characterization of relevant signalling at the cellular level (i.e. muscle fiber), as well as characterization of biochemical pathways downstream to the signals applied to the muscle fibers.

Adaptation of muscle fibers is always the net effect of the dynamics of synthesis and degradation of proteins that constitutes the force generating and passive elements of the muscle fiber. However, it should be kept in mind that in muscular adaptation not only the muscle fiber needs to adapt to new circumstances, but, in a coordinated fashion, also the basal lamina as well as collagen fiber reinforced extracellular matrix (ECM) components (the endo-, peri- and epimysial stroma of the muscle, as well as its aponeuroses).

Nevertheless, we will presently focus on muscle fiber processes.

## Protein synthesis

In a theoretical analysis of changes in the rate of muscular protein synthesis, the elements contributing to such changes may be distinguished according to two locations: outside the muscle fiber and inside the muscle fiber. The former involves changes of satellite cell activity and nucleus donation to the muscle fiber, as well as changing sarcolemmal receptor sensitivity for growth factors. The latter involves changing the quantity of mRNA and a change in the rate of translation of mRNA.

### *Extracellular events*

The number of nuclei within a muscle fiber may increase after proliferation of satellite cells and subsequent fusion with the hosting muscle fiber. Note that muscle fibers may also lose myonuclei by DNA degradation. For a given rate of transcription of mRNA per myonucleus, a change in the number of myonuclei within the sarcoplasm implicates a change in total capacity and rate of mRNA transcription.

*Activation of satellite cells, satellite cell proliferation and fusion with the hosting muscle fiber.* Within mammalian as well as amphibian muscle, the number of myonuclei per unit fiber length is proportional to the cross-sectional area of the muscle fibers (Roy et al., 1999b; Jaspers, 2002). This indicates that within healthy muscle fibers *in vivo* the volume of cytoplasm per myonucleus is strictly regulated. Such regulation was also shown after experimental hypertrophy, of rat as well as cat muscle, in response to functional overload by removal of synergistic muscles (Allen et al., 1995; Roy et al., 1999b). The satellite cells, being muscle stem cells located between the basal lamina and the sarcolemma (Mauro, 1961), are sources for additional myonuclei (Moss & Leblond, 1971). Nevertheless, induction of compensatory hypertrophy and hypertrophy in response to weight bearing at high length, is possible also without proliferation/fusion of satellite cells (Dunn et al., 1999; Lowe & Alway, 1999; Rommel et al., 2001). In partial agreement with this, Kadi et al. (2004) reported increased fiber cross-sectional area in humans because of heavy resistance training, without an increase in the number of myonuclei, despite the fact that the number of satellite cells had increased.

*In vivo*, rat and quail muscle hypertrophy in response to an increase workload or continuous weight bearing (at high length) is accompanied by proliferation of satellite cells and fusion with the hosting muscle fiber (Winchester et al., 1991; Carson & Alway, 1996; Phelan & Gonyea, 1997). The importance of satellite nuclei incorporation during hypertrophy is further indicated by the lack of compensatory hypertrophy of muscles $\gamma$-radiated prior to the hypertrophic stimulus (Rosenblatt et al., 1994; Phelan & Gonyea, 1997; Barton-Davis et al., 1999). On the basis of the literature cited we conclude that it is likely that activation of satellite cells is very important for the induction of hypertrophy. However, conditions, for which this may not be the case, deserve further research.

Particularly for postnatal growth and hypertrophy of mature muscle, several growth factors have been identified in muscle, which are likely to be involved in stimulating proliferation and fusion of satellite cells: (1) during postnatal growth, the hepatocyte growth factor (HGF) is expressed in the rat extensor digitorum muscle, but not in the mature muscle (Jennische et al., 1993), (2) weight bearing at high length enhances expression of basic fibroblast growth factor (bFGF) in the wing muscle of the chicken (Mitchell et al., 1999). (3) Immobilization of rabbit muscle at high length (Yang et al., 1997) or that in combination with increased muscle activation (McKoy et al., 1999) stimulates expression and secretion by muscle fibers of insulin-like growth factors (IGFs): IGF-1 and the IGF-1 splice variant called mechano-growth factor (MGF). The capability of such growth factors to bind to receptors on the satellite cell plasmalemma and stimulate proliferation and differentiation of muscle precursor cells has been shown for *in vitro* cultures of myoblasts and satellite cells (for comprehensive reviews see Florini et al., 1996; Grounds, 1998; Hawke & Garry, 2001; Adams, 2002; Spangenburg et al., 2002). Whether these growth factors act independently, or in synergy, remains a matter of debate.

The results of some of the *in vitro* experiments indicate the capability of IGF-1, bFGF or HGF to stimulate cell proliferation directly (Hawke & Garry, 2001). In contrast, there are also reports that IGF-1 and HGF are unable to induce myoblast proliferation themselves, but require unidentified serum components to allow myoblast division to progress (Florini et al., 1996; Grounds, 1998). These data are consistent with the finding that *in vitro* the simultaneous presence of bFGF and IGF-1 in a serum-free medium yielded remarkably higher rates of satellite cell proliferation, than when these cells were exposed to these factors independently (Doumit et al., 1993; Allen et al., 1995). Such findings indicate that proliferation of satellite cells is regulated by complex interactions between different growth factors. The feature, that may explain why the mechanisms underlying these interactions have remained undiscovered as yet, is that in order to be successful most cultures require a medium, which is supplemented with serum of unknown composition regarding growth factors and other peptides.

It should be noted that Nitric oxide synthase (NOS) producing nitric oxide (NO) is also involved in stimulating proliferation and fusion of satellite cells (Anderson, 2000; Anderson & Pilipowicz, 2002).

Myostatin, opposing effects by the factors promoting proliferation and fusion with the hosting muscle fiber, has been identified as an inhibitor of satellite cell proliferation (Thomas et al., 2000; McCroskery et al., 2003). This correlates with the finding that hind limb suspension is accompanied by enhanced myostatin expression and a substantial reduction of the number of myonuclei (Carlson et al., 1999; Kawada et al., 2001). Hypothetically, myostatin prevents replacement of degrading myonuclei, causing a reduction of the capacity and rate of the transcriptional machinery. Accordingly, myostatin may be viewed as a mediator of muscle atrophy induction. However, it should be noted that such a hypothesis was not confirmed for myostatin-deficient mice, since they lost more muscle mass during hind limb suspension than wild types (McMahon et al., 2003). Therefore, the results regarding a role of myostatin in the induction of atrophy remains ambiguous.

Therefore, it must be concluded that the absence of factors promoting satellite cell proliferation is likely to be involved in atrophy induction.

*Regulation of receptor binding of growth factors.* Heparan sulfate is one of the macromolecules of the ECM that structures water around itself and causes a less than full fluid consistency of the ECM. The sulfated glycosaminoglycan branches of heparan sulfate bind growth factors. An effect of binding of bFGF to heparan sulfate is to increase the affinity of the plasmalemma FGF receptor and bFGF for each other (Roghani et al., 1994; Lin et al., 1999). Other ligands may cause such effects as well: the effects of IGF-1 are modulated by one of six identified IGF-1 binding proteins, which affect the affinities of the IGF-1 receptor (IGFR1) and IGF-1 for each other (for a review see Jones & Clemmons, 1995; Florini et al., 1996). IGF-1 binding proteins do associate with proteins at the cell surface or further away within the ECM and by this increase the local concentrations of IGF-1 in the vicinity of the IGFR1 (Jones & Clemmons, 1995).

### *Intracellular events*

For a given number of myonuclei per muscle fiber, the rate of transcription is affected by the presence or absence of transcription factors, which either facilitate or inhibit transcription of muscle-specific genes. A higher quantity of mRNA implicates the presence of more templates for mRNA translation and hence higher capacity and rate of synthesis of the corresponding protein.

During hypertrophy of rat plantar flexor muscles in response to either increased activation or removal of synergistic muscles, MHC mRNA was increased (Periasamy et al., 1989; Wong & Booth, 1990). In addition, induction of hypertrophy of chicken anterior latissimus dorsi muscle by continuous weight bearing (at high length) was accompanied by an increase in α-skeletal actin mRNA (Carson & Alway, 1996; Carson, 1997).

The rate of translation of mRNA into peptides is determined by the number of ribosomes per mRNA as well as the rate of translation per unit mRNA.

### *Biochemical-signalling pathways involved*

The *biochemical*-signalling pathways (Fig. 2), which are likely to be involved in muscular adaptation, will be discussed briefly below.

*Regulation of transcription of mRNA.* In general, two groups of pathways are distinguished: the calcium/calmodulin-dependent pathways and the mitogen-activated protein kinase (MAPK) pathways. Both are involved in the transcriptional regulation of the adaptation of muscle size.

Calcium/calmodulin signalling. Intracellular $[Ca^{2+}]$ regulates the activity of calcineurin, a serine/threonine phosphatase and calcium/calmodulin protein kinase (CaMK) (Schulman, 1993; Klee et al., 1998). During both immobilization at high muscle length and enhanced muscle activity, the sarcoplasmic $[Ca^{2+}]$ are expected to be elevated. In the case of high muscle fiber length, $Ca^{2+}$ may enter the muscle fiber via stretch-activated channel within the sarcolemma, whereas increased activity implicates higher

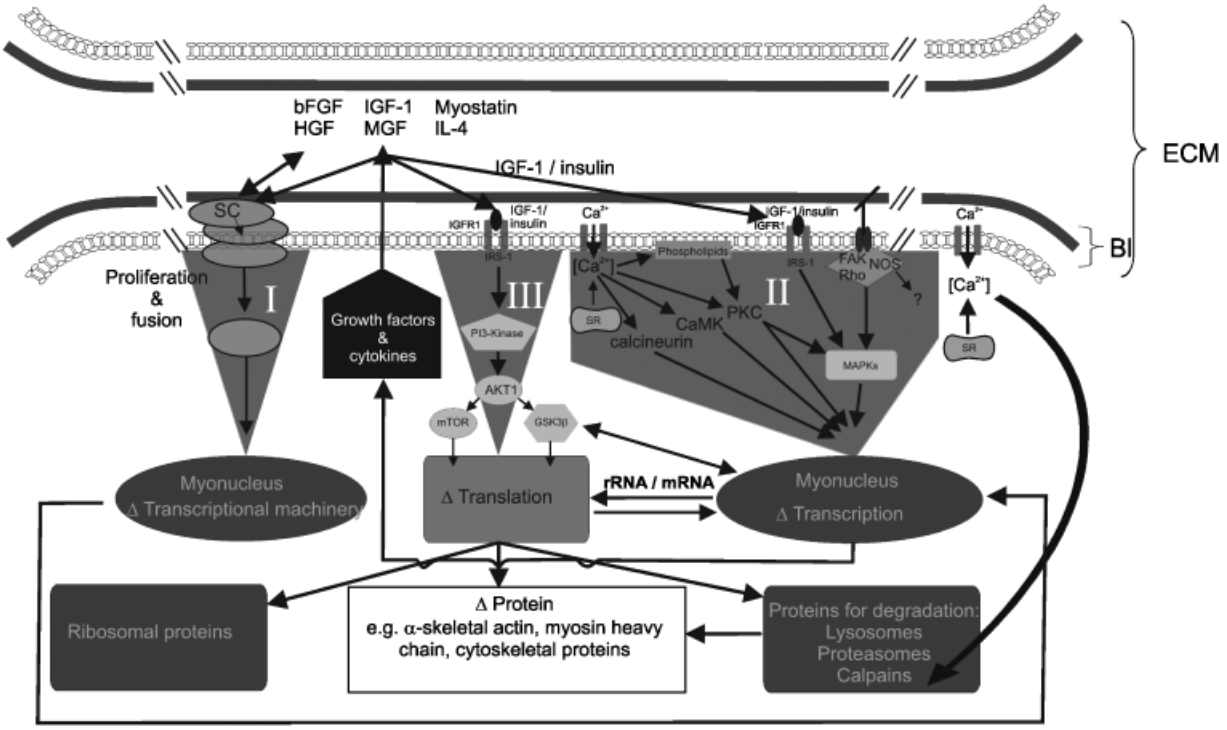


*Fig. 2.* Schematic representation of major biochemical-signalling pathways involved in the regulation of muscle size. Two neighboring muscle fibers are drawn schematically with their sarcolemma and basal lamina (BL, thick solid lines). The type I collagen fiber reinforcement (e.g. endomysium) of the extracellular matrix (ECM) is not shown for reasons of clarity (for this structure and details of BL, see Fig. 3). The abbreviations written at the location of the ECM indicate growth factors or cytokines that have been secreted there by the muscle fibers. Growth factors are proteins with hormone-like functions. Cytokines are glycoproteins (i.e. compounds consisting of mostly proteins, but with some carbohydrates as well) with such functions. Major growth factors and cytokines are: insulin-like growth factors (IGF), basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), mechano-growth factor (MGF)and myostatin, as interleukin-4 (IL-4). They bind to a number of sarcolemmal receptors and affect chemical-signalling pathways by doing so. Overviews of key pathways that are activated by effects of mechanical strain and/or increased contractile activity are shown for major effects. Note that these effects are initiated at the basal lamina and sarcolemma, the latter indicated by the chain-like elements: (I) effects on the quantity of transcriptional machinery. By fusion of satellite cells (SC) with the muscle fiber the number of nuclei is increased (cf. ''Molecular mechanisms of adaptation of muscle size''), which affects the overall rate of DNA transcription, even if this rate per nucleus is constant. (II) Given a set number of nuclei, all pathways leading to altered rates of DNA to mRNA transcription are indicated in block II. The affected transcription rates to be considered are those of mRNA related to the synthesis of muscular proteins directly (either contractile or cytoskeletal) or indirectly in two ways (a) via mRNA production for growth factors or cytokines or (b) transcription factors. This block contains the major pathways described individually in ''Molecular mechanisms of adaptation of muscle size''. Note that PKC pathways are activated by the release of calcium from the sarcoplasmatic reticulum directly or via phospholipids in the sarcolemma. For explanation of the abbreviations refer to the text in these sections. (III) The pathways affecting the translation of mRNA into proteins are grouped in block III. This block contains the pathways described individually in ''Molecular mechanisms of adaptation of muscle size''. For explanation of these abbreviations refer to the text in these sections. SR indicates the sarcoplasmatic reticulum releasing Ca ions into the cytoplasm. rRNA represents ribosomal RNA, which constitute the body of ribosomes, which are the locus of mRNA to protein translation.

or more frequent release of $Ca^{2+}$ from the sarcoplasmic reticulum.

Inhibition of calcineurin-activity-induced atrophy in mouse plantaris muscle, during compensatory hypertrophy as well as during recovery from hind limb suspension blocks the hypertrophic response (Dunn et al., 1999; Mitchell et al., 2002). This suggests that calcineurin is required for the induction of hypertrophy. The requirement of enhanced activity of CaMK in the adaptation of muscle size has not been indicated as yet, but is suggested as the activity of CaMK was increased in chicken muscle after a period of weight bearing at high length (Fluck et al., 2000).

Downstream targets of the calcineurin and CaMK-signalling pathways are transcription factors, such as the nuclear factor of activated T cells (Dolmetsch et al., 1997; Bassel-Duby & Olson, 2003), myocyte enhancer factor 2 (MEF2; Passier et al., 2000; Wu et al., 2001) and the GATA transcription factors (Musaro et al., 1999). Note that GATA represents a particular sequence of nucleotides within the DNA. Specific GATA transcription factors bind to this sequence to promote gene transcription.

Activation of calcineurin and CaMK causes conformational changes to these transcription factors, which either activate them or stimulates their translocation from the cytoplasm to the myonucleus. Both effects cause enhanced gene transcription, because activation and translocation of transcription factors facilitate their binding to promotor regions of muscle genes and by doing so enhance the rate of transcription.

Transcription of MHC and α-skeletal actin is reported to be affected by such transcription factors (e.g. Maeda et al., 2002; Sepulveda et al., 2002; Giger et al., 2004; McCullagh et al., 2004). This could be either a direct effect, or alternatively an indirect effect. The indirect effects occur through stimulation of the expression of other types of transcription factors, such as the serum response factor (SRF) and the myogenic regulatory factors Myf5, MyoD, myogenin and MRF4 (Carson & Alway, 1996; Bassel-Duby & Olson, 2003; Davis et al., 2003). This last group of transcription factors is involved in regulation of promotor activity of muscle-specific genes (e.g. Carson et al., 1995).

In addition, some of these transcription factors also enhance expression of growth factors such as IGF-1 (McCall et al., 2003) and cytokines (i.e. glycoproteins with hormone-like functions) such as interleukin-4 (Horsley et al., 2001). These growth factors are not only involved in stimulating proliferation of satellite cells, but may also affect other signalling pathways. For example, binding of IGF-1, to the IGFR1, stimulates transcription and translation of mRNA via the MAPK (see "MAPK signalling") and the phosphatidylinositol 3 kinase (PI3K)–mammalian target of rapamycin (mTOR) pathway (see "Control mRNA translation").

Whether the $Ca^{2+}$/calmodulin-activated pathways play a crucial role in the induction of muscle hypertrophy is subjecta of controversy (e.g. Dunn et al., 1999; Bodine et al., 2001b; Mitchell et al., 2002; Bassel-Duby & Olson, 2003). Inhibition of the calcineurin activity in skeletal muscle during compensatory hypertrophy or recovery from disuse-induced atrophy has been shown to block the hypertrophic response (Dunn et al., 1999; Mitchell et al., 2002). However, this finding could not be confirmed (Bodine et al., 2001b). To answer the question if these differences in experimental results are because of differing experimental conditions, further investigation is required.

MAPK signalling. Three main branches are generally distinguished for MAPK signalling:

(B1) the c-Jun N-terminal protein kinase,
(B2) the extracellular regulated kinase (ERK) and
(B3) the 38 kDa stress-activated protein kinase (p38) pathways.

Activated MAPK will phosphorylate and activate transcription factors such as c-fos, c-jun, c-myc and Elk1, as well as other kinases, which affect SRF activity and α-skeletal actin expression (Davis, 1993; Ruwhof & van der Laarse, 2000). Generally, activation of the MAPK pathways during hypertrophy is mediated via (1) receptor binding of growth factors (e.g. IGF-1) (Florini et al., 1996), (2) activation of protein kinase C (PKC) or (3) integrin (Carson & Wei, 2000) or dystroglycan signalling (Rando, 2001).

Binding of insulin or IGF-1 to their receptors results in phosphorylation of the insulin receptor substrate (IRS-1) and the Src homology containing protein (ShC), both which serve as multi-component docking platforms for proteins that contain SH2 domains. Once bound to IRS-1 or ShC, these proteins are able to activate the GTPase called Ras (McCormick, 1993), which in turn activates all three different MAPK pathways (Davis, 1993; Florini et al., 1996). *In vivo*, inhibition of the MAPK/ERK pathway within rat plantaris muscle prevents IGF-1-induced hypertrophy of muscle (Haddad & Adams, 2004), suggesting a crucial role for this pathway in the regulation of muscle size.

Rat soleus muscle can be genetically modified causing permanent expression of activated Ras (Murgia et al., 2000). The importance of MAPK activity in the regulation of muscle fiber size is further indicated by results of experiments in which such modified rat soleus muscle, that was also denervated, was prevented from atrophy (Murgia et al., 2000).

Other mediators of the MAPK activity are grouped within the PKC family (see for reviews, Jalili et al., 1999; Ruwhof & Van der Laarse, 2000) that has been shown to be involved in the induction of cardiac hypertrophy (Jalili et al., 1999). Although compensatory hypertrophy of skeletal muscle is accompanied by increased PKC activity (Richter & Nielsen, 1991), its role in the regulation of skeletal muscle fiber size is not established as yet.

Integrins and dystroglycans are trans-sarcolemmal proteins that connect the cytoskeleton and ECM of muscle fibers (see Fig. 3). Dystroglycans are known to be associated with the focal adhesion kinase (FAK) (Rando, 2001). However, there is clear evidence that binding of the extracellular domain of an integrin to ligands, such as laminin or fibronectin, stimulates the formation of the so-called focal adhesion complexes (FACs) at the cytoplasmic end of the integrin. Mechanically loading integrins stimulates activation of FAK and the small GTPase family Rho, which in turn initiate the cascade of activation of the MAPK pathways via the GTPase Ras located at the FAC (Carson & Wei, 2000; Ruwhof & Van der Laarse, 2000). MAPK activity, in rat calf muscles *in situ*, is rapidly increased in response to mechanical

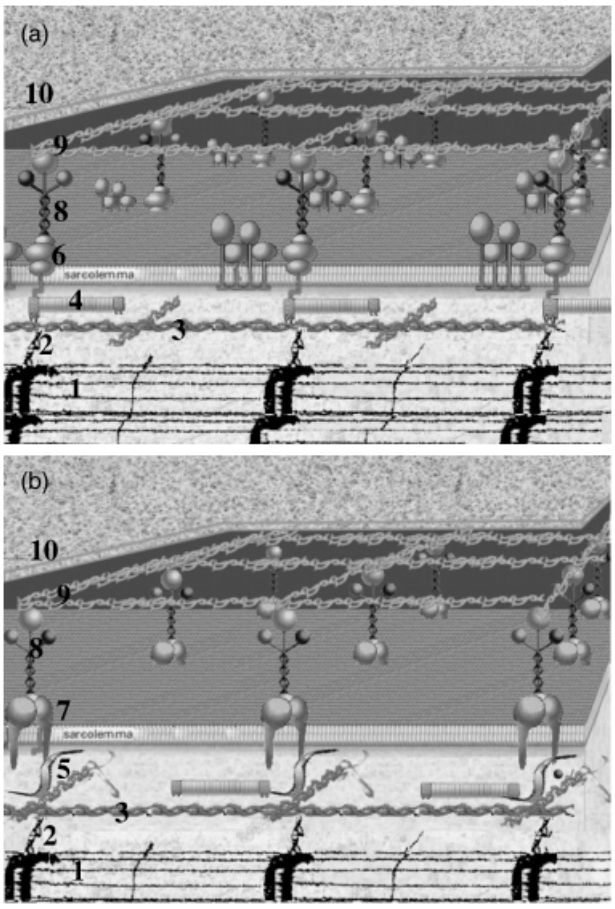


*Fig. 3*. Schematic view of supramolecular organization of connections between the cytoskeleton and the endomysium. There are two parallel systems of connections, which have the following elements in common: the cytoskeleton (1) that surrounds the sarcomeres within myofibrils and is connected by desmin filaments (2) to sub-sarcolemmal actin filaments (3). Such connections occur at least at the level of the Z-disks, but may also exist at the M line. These actin filaments (which are different from the thin filaments of the sarcomeres) are connected via two types of system-specific molecules to two system-specific types of trans-sarcolemmal molecules. These trans-sarcolemmal molecules are connected to the laminin of the basal lamina (8, which in muscle is also called merosin). Laminin is connected to collagen type IV (a non-fiber forming collagen type) of the basal lamina. The connection between the basal lamina and the collagen fibers of the endomysium (10) is made by glycoproteins. System-specific elements are: (a) the dystrophin–sarcoglycan system. For this system the connection between sub-sarcolemmal actin and laminin is made by dystrophin (4), which is connected to the trans-sarcolemmal sarcoglycans (6). (b) The talin–integrin system (also called the focal adhesion complex). Talin (5) connects the sub-sarcolemmal actin filaments via the integrins (7) to laminin. These systems are not fully independent, because, since dystrophin and talin also bind with high affinity (Senter et al., 1993), dystrophin may form a link with the integrin and dystroglycan-based systems.

stimuli (within $<10$ min) (Martineau & Gardiner, 2002). This effect is quantitatively related to passive and active tensions exerted by these muscles (Martineau & Gardiner, 2001, 2002).

Compensatory hypertrophy of rat plantaris muscle, after removal of a synergistic gastrocnemius muscle, is accompanied by enhanced activity of Rho (McClung et al., 2003). In contrast, overload of chicken anterior latissimus dorsi muscle induced by weight bearing at high muscle length was shown to increase the FAK activity (Fluck et al., 1999). Note that unloading of the rat plantar flexor muscles by hind limb suspension reduced FAK activity (Gordon et al., 2001) as well as the Rho concentrations (McClung et al., 2004). Although direct evidence for integrin-mediated MAPK signalling in the induction of hypertrophy or adding of serial sarcomeres is lacking, these findings implicate such involvement.

Recently, indications of local differences in gene expression according to location of muscle fibers within the muscle have been shown in rat medial gastrocnemius muscle in response to passive and active loading. Constant mechanical loading of the passive dissected *in situ* muscle (3 N/g muscle mass), as well as sinusoidal loading yielded fairly acutely (i.e. within 5 min) a substantially higher MAPK activity within the proximal muscle fibers than within distal muscle fibers (Csukly et al., 2002). Such local differences could not be related to local difference in muscle fiber type because other muscles (as soleus, extensor digitorum longus (EDL) and plantaris muscles) showed sensitivity of MAPK similar to the overall effect for medial gastrocnemius muscle. Instead, Csukly et al. ascribed the differences to effects on compartment tension because of local differences in muscle geometry. It should be noted, however, that substantial differences in mean sarcomeren length between proximal and distal muscle fibers are reported (Zuurbier & Huijing, 1993; Jaspers et al., 1999). These mean fiber sarcomere differences are opposite to the presumed distribution of tension over intramuscular compartments and therefore not likely to be the simple explanation of the differences in MAPK activity.

In any case, a crucial role for the MAPKs is indicated in the induction of hypertrophy. However, to answer the question if this occurs directly by stimulating the transcription of muscle-specific mRNA's or indirectly by enhancement of the expression of growth factors requires further investigation is required.

*Control mRNA translation.* The regulation of translation of mRNA involves three phases:

- initiation of translation,
- elongation of the peptide chain and
- termination of translation.

Changing the translational capacity (i.e. number of ribosomes) and/or translational rate regulates adap-

tation of muscle size. The latter of these two is particularly determined by the initiation of translation of the available mRNA and the speed of elongation of the peptide chain (Nader et al., 2002; Bolster et al., 2003).

During muscle hypertrophy in response to immobilization at high length in combination with electrical stimulation, the total RNA content increases rapidly (Goldspink, 1977; Goldspink et al., 1995). After tenotomy of rat gastrocnemius muscle, actinomycin-D blocking of RNA synthesis was reported to prevent compensatory hypertrophy of plantaris and soleus muscle (Goldberg & Goodman, 1969). As the ribosomal RNA (rRNA), which forms the basis for attachment and subsequent translation of the mRNA template, constitutes more than 80% of the total RNA, blocking the increase in total RNA would predominantly block an increase in rRNA. Accordingly, Nader et al. (2002) argued that the lack of hypertrophy by the blocking of RNA synthesis was because of a lack of rRNA synthesis capacity. An increase in ribosomal number is thought to be essential for the induction of hypertrophy. However, this rationale should be viewed with some skepticism as this argumentation only holds true when the mRNA content is not limiting the rate of translation.

In addition, several cofactors of the ribosomal proteins, such as the eukaryotic initiation factors, elongation factors and binding proteins, have been identified. These cofactors are involved in the regulation of initiation of the peptide chain as well as of the speed of elongation of the peptide chain. Regarding adaptation of muscle size, activity of these cofactors is affected by IGF-1 or insulin via the PI3K–mTOR pathway. Therefore, they are likely to be major determinants of the rate of protein synthesis (Bodine et al., 2001b; Rommel et al., 2001; Pallafacchina et al., 2002).

Details about such signalling pathways have been reviewed extensively (Shah et al., 2000; Nader et al., 2002; Bolster et al., 2003). Briefly, binding of IGF-1 or insulin to the IGFR1 at the outside of the sarcolemma recruits IRS-1, which in turn activates PI3K–mTOR pathway. Downstream target of PI3K is the protein kinase B (called PKB or Akt), which on phosphorylation activates mTOR and inhibits glycogen synthase kinase 3β (GSK3β). Activated mTOR does phosphorylate p70S6 kinase (p70S6K) as well as the binding protein for the eukaryotic initiation factor 4E (4E-BP1). When phosphorylated, both p70S6K and 4E-BP1 promote the translation of mRNA. Apart from its function in the glycogen synthesis enzyme cascades, activated GSK3β phosphorylates the eukaryotic initiation factor 2 binding protein (eIF2B) resulting in inhibition of initiation of translation. Activation of Akt, therefore, indirectly yields enhanced activity of eIF2B through inhibition of GSK3β.

Furthermore, the activation of the PI3K–mTOR pathway may also stimulate peptide-chain elongation as activation of this pathway results in decreased phosphorylation of eukaryotic elongation factor 2, which increases the speed of peptide-chain elongation. As hypertrophy in response to immobilization at high lengths and increased activity is accompanied by enhanced expression of IGF-1 (McKoy et al., 1999; Goldspink, 2003; Hameed et al., 2003) this pathway is likely to be an important determinant of the induction of protein synthesis.

Disuse-induced atrophy may also be regulated by lowering the capacity or rate of translation of mRNA. Total and phosphorylated Akt are decreased after denervation-induced atrophy. Injection of a plasmide (i.e. circular DNA) designed to permanently express an active form of AKT in denervated mouse tibialis anterior (i.e. bypassing the control of Akt) showed a substantial reduction in atrophy after denervation (Bodine et al., 2001b). In addition, muscle unloading is associated with a decrease in both mTOR (Reynolds et al., 2002) and P70S6 kinase phosphorylation (Bodine et al., 2001b), which implicate a decreased rate of translation. Other downstream regulators of translation are the inhibitory factors of translation 4E-BP-1 and EF2 kinase. The mRNA expression of these factors is elevated during different atrophy models (Jagoe et al., 2002; Stevenson et al., 2003).

*NO.* NO is a free radical produced ubiquitously by NOS. Three different isoforms of NOS have been identified (Reid, 1998; Stamler & Meissner, 2001): type I isoform (also called neuronal NOS or nNOS), type II (inducible or iNOS) and type III NOS (endothelial NOS or eNOS). Both nNOS and eNOS are permanently expressed in skeletal muscle and their activity is mediated by interaction with calcium and calmodulin (Nathan & Xie, 1994). Within skeletal muscle, nNOS is located below the sarcolemma and associated with the dystroglycan complex (Brenman et al., 1995; Gossrau, 1998). In contrast, iNOS is located in the cytoplasm and eNOS is particularly present within the mitochondria (Bates et al., 1996).

Several experiments indicate a role of NO in the regulation of adaptation of muscle size. Increased muscle activity is accompanied by increased concentrations of nNOS as is shown during electrical stimulation of the rabbit EDL and TA muscle (chronic stimulation: 3 weeks) (Reiser et al., 1997) as well as rat soleus muscle (10 min) (Balon & Nadler, 1997). In addition, reloading of rat soleus muscle after a period of hind limb unloading was accompanied by an increase in nNOS (Tidball et al.,

1998). Furthermore, release of NO from rat soleus muscle, kept *in vitro* at high length for 2 min in the presence of calcium, was significantly higher than that from muscle maintained at passive slack length at similar calcium levels (Tidball et al., 1998), suggesting that the activity of NOS is also affected by mechanical stimuli. Indications of the requirement of NO in the induction of hypertrophy and addition to the serial sarcomere number have been found (e.g. Koh & Tidball, 1999; Smith et al., 2002). Inhibition of nNOS activity during compensatory hypertrophy of rat plantaris muscle attenuates the induced hypertrophy (Smith et al., 2002). During remobilization after a period of immobilization of the muscle at low length, blocking of nNOS activity has been shown to inhibit the addition of sarcomeres in series in rat soleus muscle (Koh & Tidball, 1999).

Taken together, these findings do suggest that NOS is relevant in the induction of hypertrophy and addition of sarcomeres in series. The intracellular biochemical mechanisms via which NOS affects protein synthesis and degradation are not well understood. NO may be involved in the prevention of protein degradation.

## Protein degradation

Degradation of proteins is contrasted to their synthesis in the following way:

Synthesis involves genetic expression of the muscle proteins themselves (transcription and mRNA translation), as well as expression of cofactors and activation of those cofactors, leading to enhanced or decreased expression of the relevant muscle proteins.

Therefore, a decrease in synthesis rate of the relevant proteins at constant rate of catabolism would lead to a diminishing size of muscle.

In addition, degradation is regulated by the activity of proteolytic enzymatic pathways and by expression of cofactors and activation of these cofactors leading to enhanced or decreased expression of catalytic enzymes and their activation.

However, the susceptibility of proteins to degradation may also depend on conformational stability of the protein, which is determined by factors such as the intracellular temperature, free energy or pH.

During muscle atrophy in response to disuse or immobilization, protein degradation is enhanced by activation of proteolytic pathways (see for detailed review, Jackman & Kandarian, 2004). Three intracellular proteolytic systems are activated during muscle atrophy (Taillandier et al., 1996) and will be briefly discussed below: the calpain system, the lysosomal system and the proteasome system. However, their relative contributions to the induction of muscle atrophy in different conditions remains to be elucidated by further research.

### *The calpain system*

Calpains are calcium-activated cysteine proteases, which in muscle tend to be concentrated at the Z-disk of the sarcomeres (rat soleus muscle, Kumamoto et al., 1992). Calpains are particularly involved in the degradation of cytoskeletal proteins (Huang & Forsberg, 1998) and by doing so may make the myofibrillar proteins accessible for the proteasomes.

A period of either unloading or denervation of rat soleus muscle increases the expression of calpains within the muscle (Taillandier et al., 1996; Haddad et al., 2003). Inhibition of one of the calpains leads to substantial attenuation of the atrophy (Tidball & Spencer, 2002) illustrating their relevance in the induction of muscular atrophy.

### *The lysosomal proteases*

The organelles called lysosomes are single membrane globular systems that contain hydrolytic enzymes. Lysosomes carry hydrolases that degrade nucleotides, proteins (e.g. cathepsins and collagenases), lipids, phospholipids polymers into their monomers, and also remove carbohydrate, sulfate, or phosphate groups from molecules. These hydrolases are particularly active in an acid environment, which is fortunate because, if they leak into the cytoplasm at physiological pH ( $\approx$ 7.2–7.4), they are not likely to do much damage. Ubiquitination of sarcolemmal proteins such as receptors and channels make them targets for the lysosomal systems, which will reduce their number and thus their involvement in the induction of protein synthesis (Taillandier et al., 1996; Jackman & Kandarian, 2004).

Increased activities of cathepsin have been shown in soleus and extensor digitorum muscle from hind limb suspended rats (Goldspink et al., 1986) suggesting their possible involvement in the induction in atrophy.

### *The proteasome system*

The proteasome system is involved in protein loss in a synergistic way with the calpains. The proteasome is ubiquitous ATP- and ubiquitin-dependent proteolytic system, which is able to degrade actin and myosin *in vitro* (Solomon & Goldberg, 1996). Proteins to be degraded are modified by covalent conjugation to multiple ubiquitin molecules, which marks them for ATP-dependent degradation by the proteasome complex (Ciechanover, 1994). During different conditions of disuse of mouse muscle, such as immobilization, denervation, hind limb suspension and fasting, the expression of the recently identified muscle-specific F-box ubiquitine ligases, MAFbx and MuRF1, was were enhanced (Bodine et al., 2001a). For MAFbx, this was also the case in

fasting animals (Gomes et al., 2001). Inhibition of one of these proteasome systems leads to substantial attenuation of atrophy (Bodine et al., 2001a). Interestingly, recent data have shown that IGF-1 is a key mediator of the expression of the MAFbx and MuRF1 (Sandri et al., 2004; Stitt et al., 2004). IGF-1 activates the PI3K/AKT pathway, which in turn results in phosphorylation of the FOXO proteins, a subgroup of the Forkhead box O (foxo) family of transcription factors. Phosporylation of the FOXO transcription factors results in their cytoplasmic localization away from the target genes in the nucleus (Brunet et al., 1999), which prevents transcription of the MAFbx and MuRF1 genes. These findings indicate that IGF-1 is simultaneously able to stimulate protein synthesis and suppress protein degradation.

#### *Other mechanisms: NO*

During remobilization of mice after hind limb unloading, the soleus muscle shows sarcolemmal injury, which is accompanied by substantial increases in the number of neutrophils and macrophages (Nguyen & Tidball, 2003). As remarkably lower neutrophil concentrations were shown for muscles of transgenic mice, overexpressing nNOS during remobilization, it is suggested that NO helps to prevent enhanced workload-related membrane damage (Nguyen & Tidball, 2003) and hence a breakdown of muscle proteins. In addition, in myoblasts NO has been shown to inhibit calpain-mediated proteolysis of talin and vinculin (Koh & Tidball, 2000). This indicates that NO has the potential to increase stability of cytoskeletal proteins and by doing so diminishes the rate of protein degradation by the proteasome system (see ''The proteasome system'').

## Essentials of myofascial force transmission

Since 1983 (Street & Ramsey, 1965), it should have been clear to modern researchers that force transmission pathways, in addition to myotendinous ones, are arranged in series with the sarcomeres within muscle fibers. Only in more recent years, evidence can be found that a limited number of groups deal with this issue. If one reads much earlier literature thoroughly, it becomes clear that some people were aware of the effects of such paths, even though they did not talk about it in the specific terms of force transmission. For example Kronecker and Cash (1880) showed that muscles functioning within their natural connective tissue context are limited in their movement, compared with fully isolated muscle. Unfortunately, they decided that such constraints were not very important at sub-maximal levels of activation of the muscle and left the idea alone.

The endomysium is a tunnel-like structure that plays a major role in force transmission. A collection of such tunnels, with shared walls between adjacent tunnels, forms an integral part of the whole connective tissue stroma of a muscle. For the elegant and thought provoking images of the microscopic structure of muscle without muscle fibers see Trotter and Purslow (1992) and Nishimura et al. (1994).

Sarcomeres are connected via the cytoskeleton, trans-sarcolemmal molecules and laminin to the basal lamina. For a schematic view of molecules thought to be involved see Fig. 3. The basal lamina is connected in turn to the endomysium probably via proteoglycans (Nishimura et al., 1996). Therefore, the connective tissue stroma of a muscle (formed by the endomysia, perimysia and epimysium as collagen fiber reinforcement of the muscle ECM) is arranged in series with the sarcomeres.

Work on force transmission within muscle with non-spanning muscle fibers (i.e. muscle fibers that do not fully span the distance between the proximal and distal aponeuroses, but end within the muscle belly) has given another major push to the development of knowledge about mechanisms of force transmission by pathways other than myotendinous pathways (Loeb, 1984; Trotter, 1990; Hijikata et al., 1993; Trotter et al., 1995).

For the particular case of non-spanning muscle fibers, force transmission has been thought to occur between (parallel arranged) sarcomeres within neighboring muscle fibers, and therefore those authors (with the exception of Hijikata et al., 1993) have referred to muscle containing non-spanning muscle fibers as series fibered muscle.

However, we have to consider the likeliness that most of the force is transmitted predominantly through the muscular connective tissue stroma rather than onto the adjacent muscle fiber. In such a case non-spanning muscle fibers would be arranged in parallel rather than in series. If shearing of the basal lamina and endomysial interfaces would not make the interface with the endomysium very stiff, very little of the force would be transmitted via such a pathway even in otherwise extreme conditions. In such conditions with tenotomy of a fraction of muscle fibers from a fully dissected muscle (preventing myotendinous force transmission for the afflicted muscle fibers), the force would be expected to fall according to the decrease in physiological cross-sectional area with tendinous connections at both ends of the muscle fibers. This was shown experimentally not to be the case (Huijing et al., 1998). In addition, if the considerable force still exerted by the afflicted muscle fibers would have been transmitted onto the active sarcomeres of the neighboring muscle fibers, having unaltered myotendinous function at both fiber ends, the lengths of those sarcomeres and

thus of those muscle fibers would have to be increased substantially by the additional load imposed on them. It was shown experimentally that this did not occur (Huijing et al., 1998). Therefore, we conclude that most of the force that is transmitted by shearing of the basal lamina and the interface between the groups of muscle fibers is transmitted further via the muscular connective tissue stroma and that is the reason why we should refer to the whole process as myofascial force transmission.

The alternative correct way of expressing this is that for a target sarcomere the myofascial connections form an additional load to be borne. Such an additional load would prevent a sarcomere from shortening sooner than in the case where only serial sarcomeres loaded a specific target sarcomere.

## Epimuscular myofascial force transmission

In almost all physiological experiments *in vivo*, *in situ* and *in vitro*, muscular force or its equivalent (e.g. moment) is measured exclusively at one end of the muscle (or at one joint). Implicitly, it is assumed that the origin and insertion of the muscles (tendinous or otherwise) are the only structures arranged in series with the sarcomeres of the muscle: in such a case proximally and distally exerted forces would be identical. The arguments presented above have led us to initiate simultaneous measurement of force at proximal and distal tendons of muscle, where possible. Results of those experiments have led to a major change in our views on muscular function. The main visions of muscular function derived from experimental as well as modelling results regarding such conditions will be presented below.

If force is transmitted from the muscular stroma to anything else other than the muscles own origin or insertion tendons, we speak of epimuscular myofascial force transmission. An alternative way to express the same condition is to say that the connective tissues outside the muscle of interest may constitute an additional load on all or a fraction of the sarcomeres within the muscle fibers.

### *Characteristic feature: proximo-distal force differences*

In all conditions with any net epimuscular force transmission, forces exerted at origin and insertion of a muscle or a muscle fiber are not identical (Huijing & Baan, 2001a). The additional load imposed by epimuscular connective tissue structures will affect forces exerted at specific tendons. Figure 4 shows an example of such force difference for a tetanic contraction of rat EDL muscle that is active within its natural connective tissue context. It should be noted that an extremely important variable, in addition to muscle length, is muscle position relative

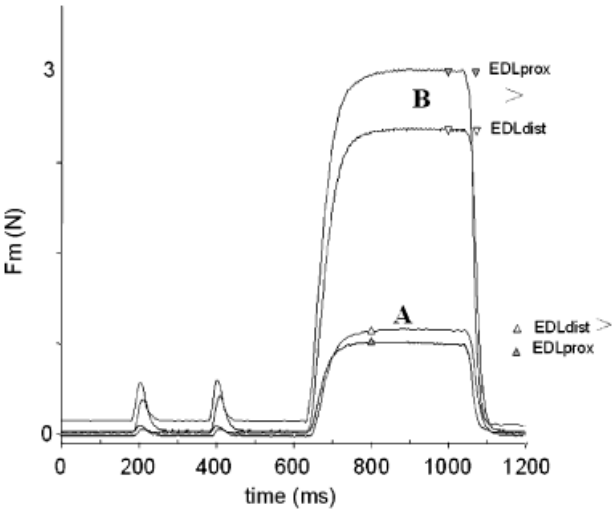


*Fig. 4.* An example of proximo-distal force differences in rat extensor digitorum longus (EDL) muscle. Force–time traces of EDL isometric contractions are shown at two lengths. (1) At low lengths EDL exerts a small force (a), but the amplitude of the distal force dominates that of the proximal force. This occurs in both twitches seen at 200 and 400 ms, as well as during the plateau of the tetanic contraction. (2) At a high length, obtained by proximal lengthening, EDL force is much higher (b), but in this case the proximal force dominates the distal force, i.e. the side of dominance of force is reversed.

to surrounding tissues. Proximal or distal lengthening to the same muscle length yielded quite different effects on the forces exerted at proximal and distal tendons, as well as on the proximo-distal force difference (Huijing & Baan, 2003). For distal lengthening, distal force increased more than proximal force after proximal lengthening of EDL. The sign of the proximo-distal force difference reversed for proximal compared with distal lengthening. Also, for distal lengthening, the proximo-distal force differences were higher.

The specific effects of relative position can be shown more clearly if a muscle at constant length is moved through its connective tissue context (Maas et al., 2004): depending on position, the proximo-distal force difference changed not only in magnitude, but also in sign!

### *Two types of epimuscular transmission*

Two types of epimuscular myofascial force transmission are distinguished:

*Intermuscular myofascial force transmission.* If the myofascial load on the sarcomeres within the fibers of a muscle is imposed via the direct and very short connections of two adjacent muscular connective tissue stromata, we speak of intermuscular myofascial force transmission. In such a case shearing of the shared epimysium may yield the stiffness that allows

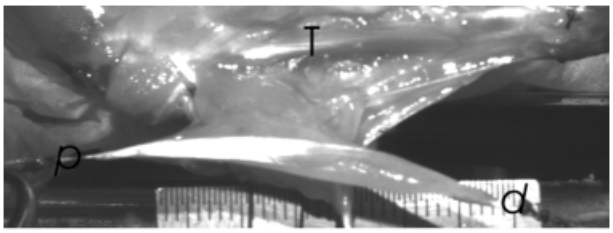


*Fig. 5.* The neurovascular tract as seen in a lateral view of the dissected rat lower leg. If one loads a dissected extensor digitorum longus (EDL) at proximal and distal tendons (i.e. in vertical direction of the image), as the EDL is pulled down, a connective tissue sheet is exposed that connects the intramuscular connective tissue stroma of the EDL (and the other muscles, not shown) to other passive elements of the anterior crural compartment. As this sheet envelops both nerves and blood vessels that either enter the muscles of the anterior crural compartment or continue on into the foot or into the peroneal compartment we refer to it as neurovascular tract. It should be noted that *in vivo* the neurovascular tract does not necessarily have the shape of a sheet. Connections of this tract are made along its full length to the interosseal membrane, the anterior intermuscular septum and the periost of the tibia. The tract also forms a connection to the peroneal compartment and its muscles as it passes through a fenestra (or window) in the intermuscular septum (see also Fig. 6). The smallest divisions on the ruler indicate millimetres.

force transmission. If such intermuscular connections are stiff enough, force that has its origin within sarcomeres of one muscle may be exerted via the direct stroma–tendon connections at the tendon of an adjacent muscle.

*Extramuscular myofascial force transmission.* If the myofascial load on the sarcomeres within the fibers of a muscle is imposed via non-muscular connective tissue elements, we speak of extramuscular myofascial force transmission between sarcomeres of a muscle fiber and the extramuscular structures.

A major extramuscular structure that may be involved in this pathway is the neurovascular tract, i.e. the collagen fiber reinforced sheet or bundle of connective tissues that envelops and protects blood vessels, lymph vessels and nerves and their braches outside the muscle (Fig. 5). The sheet or bundle is continuous with the muscular stroma along most of the length of the muscle belly, as the continuously branching nerves, blood and lymph vessels within the muscle are embedded within the peri- and endomysia. However, at specific locations, major branches of blood vessels and nerves enter the muscle belly. At the other extremity of the sheet, the neurovascular tract may also be attached to structures (i.e. intermuscular septa, interosseal membrane and periost) forming the walls of a compartment in which a muscle group is organized. It should be realized that such structures are, sometimes very directly, connected to the capsule and ligaments of the joint (Fig. 6), so that some of the extramuscularly transmitted force may be used for stabilization of the joint.

In most physiological experiments on muscle, the muscles are fully dissected from their surroundings with the exception of their innervation and blood supply, the integrity of which is usually crucial for the experiment. This means that in the so-called *in situ* experiments at least a remnant of the neurovascular tract remains. Such a remnant was shown to be capable of maintaining a substantial proximo-distal force difference for passive, but not for active force (Huijing & Baan, 2001a). However, it has since been shown that the existence of a proximo-distal active force difference is dependent on the relative

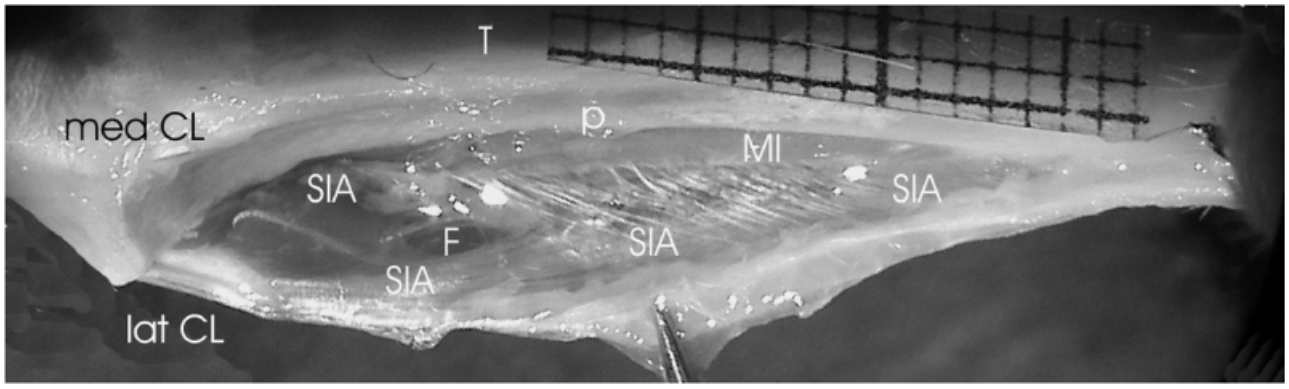


*Fig. 6.* Connections of passive elements of the anterior crural compartment to ligaments of the knee, in addition to the general fascia. The major connective tissue structure shown in this image of the fully dissected rat anterior crural compartment is the anterior intermuscular septum (SIA) with its fenestra (F). The SIA is being strained via the forceps that is pulling on dissection remnants of the general fascia seen in the bottom. SIA is attached at almost right angles to the interosseal membrane, which runs between the tibia (T) and the fibula below the tibia in this image (not to be seen). Note that at the bottom left the SIA is supported by the lateral collateral ligament that crosses the knee joint. The tibia is surrounded by its own membrane, the periost (p), which is also connected to the medial collateral ligament (med CL) of the knee. Note that a part of any force exerted via the neurovascular tract onto these structures may be exerted at the knee.

position of the "fully dissected muscle" with respect to the neurovascular tract and other tissues (Rijkelijkhuizen et al., 2004; Yucesoy et al., 2005).

It should also be noted that the neurovascular tract forms an indirect connection between the stromata of two adjacent (i.e. synergistic) muscles. Similarly, neurovascular tracts even form a connection between bellies of (antagonistic) muscles located in adjacent compartments.

Recent evidence indicates that active force may even be transmitted via extramuscular myofascial pathways to the insertion of a muscle on bone via epitendinous connective tissues (Rijkelijkhuizen et al., 2004).

Recently, for some human cadavers, Bojsen-Møller et al. (2004) reported a rare anatomical variation consisting of aponeurotic connections between distal aponeuroses of gastrocnemius and soleus muscle. Similar connections are also known between some of the distal tendons of human extensor digitorum communis muscle, but they seem to occur more frequently there. Even though these connections can yield mechanical interaction between the connected muscles, they should not be confused with myofascial connections, since the connections occur along the myotendinous rather than the myofascial paths.

#### *Epimuscular myofascial force transmission between adjacent synergists*

For conditions involving several muscle groups activated and kept at constant muscle tendon complex lengths, lengthening of one muscle or complex of muscles affects force exerted at their origin and insertion by adjacent muscles within the compartment. This is a rather general feature (Maas et al., 2001, 2003a; Huijing, 2002; Huijing & Baan, 2003; Huijing et al., 2003; Yucesoy et al., 2003). Figure 7a, b shows an example of such results. In that case, length–force characteristics of the tied rat tibialis anterior and extensor hallucis longus complex were determined. Length and position changes of that complex caused substantial changes in the proximo-distal active force difference of EDL, which was kept at constant muscle–tendon complex length. This indicates changes in epimuscular myofascial force transmission between EDL and surrounding structures within the anterior crural compartment.

#### *Substantial serial distribution of sarcomere length expected*

Intuitive reasoning leads to an expectation of serial sarcomere length distributions. Any active sarcomere will shorten to its active slack length if unopposed by an external force (load) that balances the sarcomere force. The active slack length is defined as the length at which the sarcomere is active, but cannot exert forces to its outside. The load imposed on the sarcomere by the origin and insertion will definitely act as such an opposing force. However, the epimuscular load is, like the myotendinous load, arranged in series with the sarcomere and thus will contribute to the force that will equilibrate with the force exerted by the contracting sarcomere.

It should be noted that the stiffer parts of the neurovascular tract (i.e. the locations where the nerves and blood vessels do enter the muscle) are not distributed uniformly over the muscle belly, but are found at specific locations. As a consequence, the epimuscular load is distributed over the muscle stroma, and the loads will not be distributed uniformly to all sarcomeres within the fibers of the muscle. Therefore, the sarcomeres exposed to a lower load will shorten more before isometric force equilibrium can be reached than the sarcomeres that are more heavily loaded.

### Force transmission between antagonistic muscles

The question posedis whether such intra-compartmental effects of interaction between muscles will also be present for muscles located within different compartments (Huijing, 2003). Potential myofascial connections between antagonistic muscles are per definition only of an extramuscular nature, because there is no direct contact between the stromata of antagonist muscles. This illustrates that intermuscular myofascial effects can in principle also be mediated by extramuscular tissues.

The reasoning behind such hypotheses regarding myofascial interaction between antagonistic muscles is based on the fact that the relative positions of antagonistic muscles change most drastically of all. If extramuscular myofascial connections of sufficient stiffness exist between antagonistic muscles, we should expect very substantial effects. Figure 7a, c shows preliminary experimental results indicating that this is indeed the case. As force exerted by the rat tibialis anterior and extensor hallucis longus muscles at their tied together distal tendons was determined at progressively higher lengths, distal force exerted by the antagonistic peroneal group dropped progressively to as much as 25% of the initial force, despite the fact that the muscle tendon complexes of the peroneal group were kept isometrically.

It should be noted that during *in vivo* movement the relative positions of the two muscle groups changes even more than in the experiment performed because both synergistic and antagonistic muscle will change their length in opposite directions, so that at the extremes of the movement range one should expect very sizable effects.

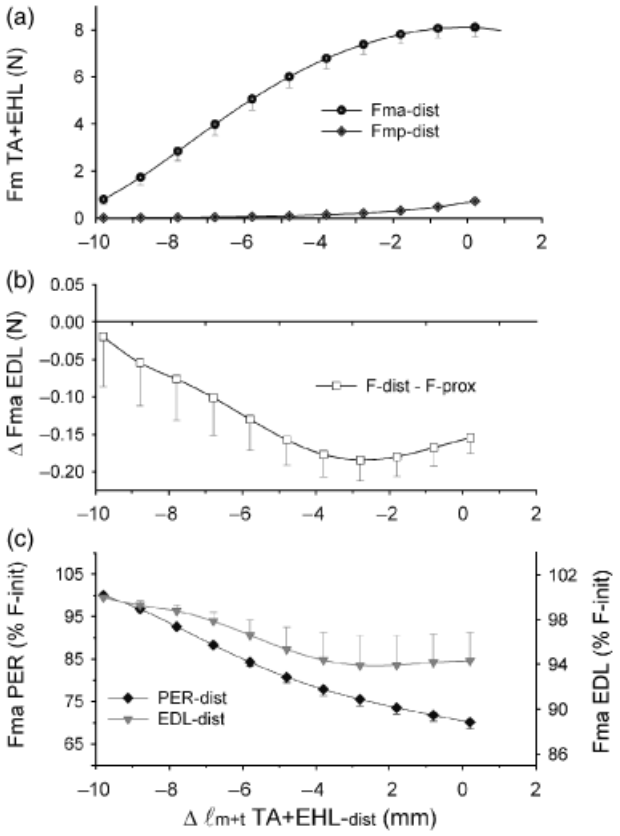


*Fig. 7.* Extramuscular myofascial force transmission between synergistic and antagonistic muscles: (a) length force characteristics (active and passive) of the rat tibialis anterior and extensor hallucis longus muscles of which the distal tendons have been tied together (TA+EHL). Note that the length of this complex was exclusively altered in this experiment. (b) The proximo-distal force difference of rat EDL (synergist of TA and EHL) and the effects of changing TA+EHL length on it. Note that as TA+EHL is active at higher lengths, the proximo-distal force difference first increases its negative amplitude and then decreases it, despite the fact that EDL muscle–tendon complex length was unchanged. (c) Force exerted by all peroneal muscles (PER), while being kept at constant muscle-tendon complex length, at different lengths of antagonistic TA+EHL. Force is expressed as percentage of initial force (at low TA+EHL length). Note that as TA+EHL length and active force increase, PER active force decreases by as much as 30% (use left *Y*-axis), despite being kept at constant length. A similar feature is seen for EDL distal active force, but the amplitude of that decrease is limited to about 6% (use right *Y*-axis in grey). TA+EHL length is expressed as a deviation from its optimum length and the *X*-axis applies to all plots of this figure.

In several different experiments in our laboratory (unpublished observations), we have found similar effects and interactions for all antagonistic muscle groups within the rat lower part of the hind limb. From such results, we conclude that even antagonistic muscles should not be considered as being independent of each other and that by specifying experimental conditions regarding adaptation of muscles and tendons we should consider exclusively not only the condition of the target muscles, but also the conditions of all other muscles within the same segment. Since several muscles related to a particular segment are bi- or even poly-articular, this also means that the conditions at joints further removed from the segment of interest should be taken into account when specifying experimental conditions of a specific target muscle regarding muscular adaptation and its effects.

It may be this factor that has added to the difficulty of interpreting mechanisms of adaptation active in classical experiments. Therefore, it is concluded that the usual classical experimental conditions are too complex to permit an enhanced understanding of the real determining factors, let alone the mechanisms of adaptation. It is clear that if we want to attain goals described in the introduction a new approach to the study of muscular adaptation of serial sarcomere

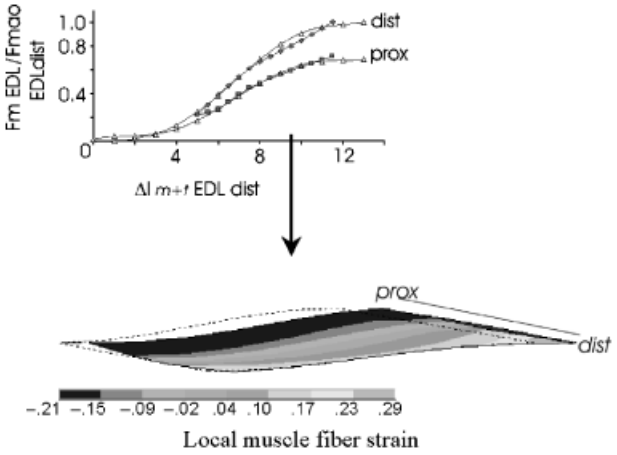


*Fig. 8.* Combined experimental and finite-element model results for distal lengthening of rat extensor digitorum longus (EDL) with epimuscular myofascial connections. The top panel shows experimental length–force curves of rat EDL muscle, as well as a modelled segment of that curve. Distal lengthening was used to change the EDL length. EDL length is expressed as a deviation ($\Delta lm+t$ dist) from initial low length. Force was normalized for optimal distal EDL force ($F_{\text{mao EDLdist}}$). In the specific conditions of these experiments, at all modelled lengths, distal force (dist) dominates proximal force (prox). Solid markers indicate model force. For the length indicated (arrow), the lower panel presents the distribution of strain in the muscle fiber direction (indicated by a solid line with marking for proximal fiber end (prox) and distal fiber end (dist)). The strain value represents the fractional change from the initial length (e.g. −0.15 represents a 15% shortening with respect to initial conditions). Note the particularly high serial distribution of strains within the model, indicating a high serial distribution of sarcomere lengths within muscle fibers. Note also that the lower proximal force is related to shortened sarcomeres, not exposed to the added epimuscular myofascial load that allows distal sarcomeres within the same muscle fibers to be at higher lengths. The dotted contour indicates the starting condition of the muscle.

number and physiological cross-sectional area must be developed.

### Finite-element models of epimuscular myofascial force transmission confirm relatively high serial and parallel distribution of muscle fiber strain

Since during myofascial experiments, the connective tissue around the muscle belly was left intact as much as possible we cannot study details of the muscle, because we cannot visualize them. Finite-element models have helped us to understand what happens within the muscle in conditions where epimuscular myofascial force transmission is evident from simultaneous force measurements at both proximal and distal tendons. Figure 8 shows an example of combined experimental and model results. For details of such modelling and experiments, see Yucesoy et al. (2003). A clear proximo-distal total force difference is present in rat EDL muscle at a length over optimum length. Calculated local muscle fiber strains (represented as the fraction of actual lengths over the initial lengths) yield an estimate of local sarcomere lengths. Despite the high length of the muscle, particularly at the extramuscularly connected face of the model, sarcomeres located near the proximal end of muscle fibers are shortened by up to 14% below their initial length, whereas sarcomeres closer to the other (distal) end of the same muscle fiber are lengthened by up to 50%. The distal sarcomeres are lengthened much more because extra- and intermuscular myofascial forces load them additionally. It is clear that such myofascial connections cause high and stable serial distributions of sarcomeres, which lead to the exertion of different active and passive forces at the proximal and distal end of each muscle fiber.

Such phenomena have very many functional consequences. For example, intersarcomere dynamics are not exclusively interactions between only sarcomeres in series, as always assumed in the literature (e.g. Denoth et al., 2002; Telley et al., 2003), but actually constitute interactions between new units entities arranged in series. This new unit is constituted by a sarcomere together with its relevant part of the endomysium of the muscle fiber (arranged in parallel to the specific sarcomere). Serial interaction between such new units is much more stable mechanically because of the parallel paths of force transmission. Therefore, myofascial force transmission is very likely to prevent the so-called popping sarcomeres, which have been presumed to occur (Morgan, 1990) because only the sarcomeres were considered as exclusive serial units.

Also for adaptation, this finding is highly relevant, because conditions will be very different for different parts of the same muscle fiber. This means that the ostensibly relatively simple conditions imposed on a muscle during the classical experiments on adaptation are actually very complex, because the conditions even differ locally along the length of one muscle fiber.

It should be noted that, for *in vivo* magnetic resonance imaging (MRI) measurement of strain and displacement velocity of aponeurosis and muscular elements within human muscle, considerable distributions of the values of these variables are reported (Pappas et al., 2002; Finni et al., 2003a, b) within a muscle. Such results are very difficult to explain without the concepts of epimuscular myofascial force transmission. In any case they at least confirm the *in vivo* presence of the types of distributions foreseen on the basis of the finite-element model calculations. More specific modelling of these *in vivo* results is indicated to aid their interpretation.

### Different species and epimuscular myofascial force transmission

Epimuscular myofascial force transmission has been shown to exist and have substantial effects in mammalian muscle both in rodents Huijing, 1999, 2002; Huijing & Baan, 2001b, 2003; Maas et al., 2001, 2003a–c, 2004; Smeulders et al., 2002; Huijing et al., 2003; Yucesoy et al., 2003; Rijkelijkhuizen et al., 2004) and in human patients suffering from spastic paresis (Smeulders et al., 2002, 2003, 2004a, b, c; Kreulen et al., 2003).

Below we will present evidence regarding adaptation in amphibian muscle fibers. Therefore, it is necessary to ascertain that perimuscular myofascial force transmission is also active in these species. Presently, we are only able to present some preliminary data on *Xenopus laevis* muscle. However, such data are essential for the line of reasoning of this article.

#### *Summary of methods used in X. laevis force transmission experiments*

The experimental approach was similar to that used for rats: the animal was anaesthetized using urethane solution (dose 0.05 mL/g body mass of 12.4% solution). Over the target muscle, skin and subcutaneous tissues were removed and fasciotomy was performed on the anterior tibial compartment. A muscle with both proximal and distal tendons was selected: in this case TA muscle with one tendon crossing the knee joint and two distal tendons crossing the ankle joint. A force transducer was attached to both the proximal tendon and the combined distal tendons. The ankle was maximally plantar flexed to allow free passage of the distal TA tendons and their connections. High in the femoral compartment, the distal end of the cut sciatic nerve was stimulated maximally at 80 Hz: all

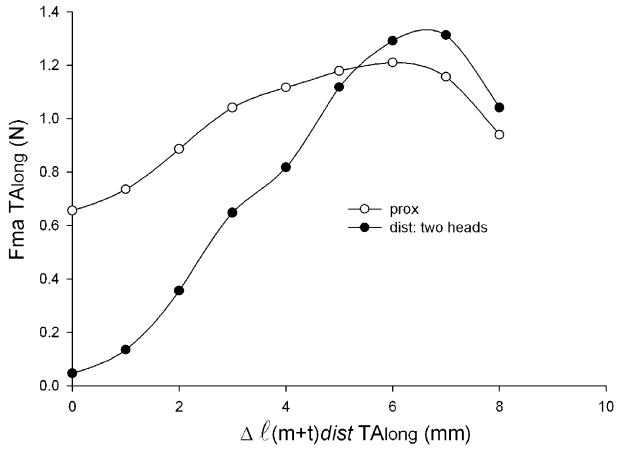


*Fig. 9.* An example of length–force characteristics of *Xenopus laevis* tibialis anticus longus muscle embedded within its natiural connective tissue context. Active force exerted by maximally active TA was measured at its proximal tendon, as well as the summed forces exerted by its two heads at the distal two TA tendons that were connected to one force transducer. Note that the proximo-distal active force differences observed are particularly high at low lengths, with proximal force dominating distal force. At length higher than the crossover point (approximately $\Delta l = 5$ mm), distal force dominates proximal force but with smaller differences. The presence of such force differences indicates epimuscular myofascial force transmission.

muscles in the lower leg were simultaneously active maximally. Ambient temperature was regulated at 22 °C.

#### *Preliminary results*

At all muscle–tendon complex lengths studied, but one, a proximo-distal force difference was found: at low lengths considerable distal active force was exerted (>20% of distal optimal force), but simultaneously proximal active force equalled zero (Fig. 9). At progressively higher lengths obtained by distal lengthening, proximal as well as active distal force increased, but not in a similar fashion: distal active force increased much more. As a consequence, force values approached each other, and after further lengthening the proximo-distal force difference reversed its sign, i.e. distal force became dominant over proximal force. These results are very similar to those of previous experiments in other species and indicate that epimuscular myofascial force transmission is also active in *X. laevis* muscle.

### Some important consequences of myofascial force transmission for the study of adaptation

The conditions imposed on the muscle fibers of a muscle that is working within the natural context of its epimuscular connective tissues are quite complex. The serial sarcomere length distribution within muscle fibers is dependent not only on the length of the muscle fibers, but also on the relative position of those fibers with respect to other muscles and connective tissues within the limb.

Direct intervention with the connective tissues, as in ablation of synergistic muscles, will change the conditions of myofascial force transmission drastically. Simple fasciotomy of the compartment as well as progressive dissection of muscles and tendon already does that (Huijing & Baan, 2001a; Smeulders et al., 2002; Huijing et al., 2003; Kreulen et al., 2003; Rijkelijkhuizen et al., 2004). Release of a retinaculum does not only change the length and length range of a muscle of which tendons pass through but it will also change the relative position of the muscle with respect to connective tissues of a compartment as well as to other synergistic and antagonistic muscles.

Simple tenotomy will not remove a muscle from functioning because of myofascial force transmission, because the muscle will not shorten to its active slack length (Kreulen et al., 2003), but will be restrained at higher lengths and may even be lengthened by surrounding tissues.

The serials distribution of sarcomere length within muscle fibers, enhanced by myofascial effects, raises doubt on the concept, introduced above, that the condition of the muscle fiber as a whole will be the determining factor of adaptation. Considering the asymmetries in muscle length effects (e.g. much more powerful adaptation effects of high length compared with low lengths: Williams, 1990), adaptation of muscle fiber size may be a much more local affair involving only parts of the muscle fiber. It is conceivable that, regarding adaptation, the muscle fiber as a syncytium should not be considered as one unit, but rather as a collection of units of adaptation, each of which react to its own local-specific mechanical stimuli.

Therefore, it is also possible that the specific myofascial effects on a muscle fiber will be responsible for signals of adaptation. It is clear that to be able to get closer look at the detailed mechanisms of muscular adaptation we need to create experimentally mechanical conditions which are less complex than those usually afforded during *in vivo* experiments.

## Study of adaptation at the cellular level

Unravelling the mechanisms of adaptation of muscle size requires a sophisticated *in vitro* approach to overcome the problems related to the substantial distributions of sarcomere strains expected within muscle and muscle fibers during *in vivo* conditions.

Several *in vitro* setups have been developed for mature mammalian, single muscle fibers. Since their

initiation in the forties of the last century, such systems have primarily been used for acute investigation of physiological and mechanical properties of amphibian muscle fiber (e.g. Ramsey, 1947; Natori, 1954; Gordon et al., 1966; Lannergren, 1978; Westerblad & Lannergren, 1986; Van der Laarse et al., 1991).

However, more recently they have also been used to study acute effects of hormones and growth factors on cytoplasmic [$Ca^{2+}$] (Bruton et al., 1999). In addition, long-term cultures of single mature muscle fibers have also been developed and used to study [$Ca^{2+}$] homeostasis (De Backer et al., 2002), the effects of growth factors on satellite cell proliferation (Bischoff, 1986) as well as MHC gene expression in response to different activation regimes (Liu & Schneider, 1998). Such *in vitro* systems, however, are inappropriate for the investigation of long-term adaptation of muscle fiber size, because muscle fibers are maintained in the presence of serum and fiber length cannot be manipulated as the muscle fibers are released from the tendons during the isolation by using catalytic enzymes that facilitate cell isolation (Bischoff, 1986; Liu & Schneider, 1998). In addition, these enzymes may cause damage to or removal of the endomysium (Bischoff, 1986) and basal lamina, as possibly was the case with Liu and Schneider (1998), which may affect both the ligand-receptor signalling and the mechanical signalling via trans-sarcolemmal complexes.

Most of our knowledge, regarding (1) how mechanical signals affect cells and (2) how the signals are subsequently converted into intracellular biochemical activities that regulate protein synthesis and degradation, was based, until recently, on results of culture experiments using myoblasts or non-muscle cells such as epithelial cells, fibroblasts or osteoblasts. These types of cells are morphologically and functionally different from mature muscle fibers and, generally, culture of these types of cells needs to be performed in the presence of a serum, of unspecified content, to maintain the cells viability. Despite the limitations of these models, such experiments have substantially enhanced our understanding of fundamental mechanisms of cellular signal transduction.

Two pathways of mechano-transduction are distinguished: (1) mechano-chemical signal transduction to the nuclei, i.e. an indirect pathway by which mechanical stress applied to the muscle–tendon complex is transmitted onto the ECM including the endomysium to trans-sarcolemmal structures of muscle fiber and subsequently converted at that location into biochemical signals as treated above and (2) mechano-transduction, i.e. pathways by which the mechanical load exerted on the endomysium–basal lamina–trans-sarcolemmal complexes of the muscle fiber is transmitted via the cytoskeleton onto the myonucleus and leads more directly to gene expression.

## Mechano-chemical transduction

Within cell membranes, several trans-membrane receptors and channels are recognized that are able to elicit biochemical activities upon mechanical loading. Within muscle, the trans-sarcolemmal proteins most likely involved in the adaptation of muscle size are the integrins, the dystroglycans and the stretch-activated calcium channels (for reviews, see Carson & Wei, 2000; Ruwhof & Van der Laarse, 2000; Rando, 2001). Among these, the integrins and dystroglycans are the core proteins of adhesion complexes that connect the ECM and the cellular cytoskeleton. Pardo et al. (1983) first recognized the connection of the myofibrils to the sarcolemma. Because of their shape of the immunofluorescence they called these sites ''costameres''. Later, the costameres were shown to be sites of transmission of myofibrillar force of adult cardiac myocytes to the ECM (Danowski et al., 1992) and were hypothesized to reinforce and stabilize the sarcolemma in skeletal muscle fibers (Petrof et al., 1993). Subsequently, these trans-sarcolemmal complexes were shown to be also involved in activation biochemical-signalling pathways.

### *The integrins*

The integrin family can be described as heterodimeric transmembrane glycoproteins consisting of $\alpha$ and $\beta$ subunits. Binding the integrin to glycoproteins in the ECM, such as fibronectin and laminin, results in the formation of sub-sarcolemmal FACs within the muscle fibers, in which vinculin is thought to provide a major mechanical linkage (Fig. 3) between the integrin and the subsarcolemmal actin filaments (different from sarcomeric actin filaments), which are part of the cytoskeleton (Berthier & Blaineau, 1997). Also bound to vinculin is talin (Fig. 3). Loading of FACs stimulates translocation and accumulation at these sites of kinases, small GTPases and other signalling molecules (e.g. PI3K, FAK and Rho) (for reviews, see Ingber, 1997; Janmey, 1998; Carson & Wei, 2000). These signalling molecules are able to activate several signal transduction pathways such as the MAPK and the PI3K–mTOR pathway, which in turn increase both mRNA expression and translation (see ''Biochemical-signalling pathways'').

### *The dystroglycan complexes*

In addition to the integrins, the dystroglycans also provide a mechanical linkage between the ECM and the cytoskeleton (Berthier & Blaineau, 1997; Rando, 2001). Whereas vinculin is indicated as the major protein that connects the integrin to the cytoskeleton,

for the dystroglycans, dystrophin is identified to form the connection to the cytoskeleton. Like for the integrins, the dystroglycan complexes are also assembly points for signalling molecules such as FAK and RhoA, which have been shown to be able to mediate biochemical-signalling pathways affecting the rate of protein synthesis (Berthier & Blaineau, 1997; Rando, 2001).

Evidence of the involvement of the dystroglycan complexes in mediating biochemical-signalling pathways such as the MAPK, the NO and the PI3K–mTOR pathways is emerging (Rando, 2001; Langenbach & Rando, 2002; Spence et al., 2004). The finding that dystrophin knockout mice do not express MGF (Goldspink et al., 1996) also indicates involvement of the dystroglycan system in the induction of muscle hypertrophy. MGF is a potent stimulator of proliferation of myoblasts (Yang & Goldspink, 2002) and therefore is likely to play an important role in the activation of satellite cells. The precise mechanisms of dystroglycan complexes involvement in adaptation of muscle size remain to be determined.

#### *Linking of the integrin and the dystroglycan systems*

As dystrophin and talin bind with high affinity (Senter et al., 1993; Yoshida et al., 1998), dystrophin also plays a role in linking both costameric trans-sarcolemmal systems. Consequences of connecting these two systems for force transmission and the pathology of Duchenne muscular dystrophy (i.e. dystrophin deficiency) have not been considered as yet. In contrast, regarding signal transduction bi-directional communication between the dystrophin-containing complex and the integrin adhesion system has been shown in cultured myocytes (Yoshida et al., 1998). Further work on this topic is indicated.

#### *Stretch-activated channels*

Another type of mechano-sensitive trans-sarcolemmal structure is the stretch-activated channel. Stretch of cardiac myocytes causes direct influx $Ca^{2+}$ via these channels (Gannier et al., 1996; Tavi et al., 1998), which likely activates the $Ca^{2+}$-mediated pathways as mentioned above.

At higher lengths, mature isolated frog muscle fibers show a length-dependent increase in free intracellular [$Ca^{2+}$], starting at a mean sarcomere length of 2.4 μm (Snowdowne, 1986). Stretch-activated channels are also detected in skeletal muscle fibers (McBride et al., 2000) and may mediate elevation of intracellular [$Ca^{2+}$]. Whether the stretch-activated channels are involved in the induction of skeletal muscle hypertrophy remains to be clarified. With respect to this, it should be noted that the stretch-activated channels colocalize with a system of connection to the cytoskeleton (i.e. the spectrin system, Berthier & Blaineau, 1997).

### Mechano-transduction

The alternative way by which mechanical load exerted on cells is transduced into changed protein synthesis or degradation is based on the concept that cells are hard-wired systems according to the principles of tensegrity. Combinations of stiff rods and pre-stressed elastic strings characterize tensegrity structures that yield stability. Movement of one element of a tensegrity system involves movement of the integral structure. Ingber (1997, 2003a, b) has postulated the model of tensegrity-based signalling in biological cells. This model proposes that the cytoskeleton inclusive costameres constitute a pre-stressed tensegrity structure that allows force transmission from the collagen fiber reinforced ECM onto the cytoskeleton and nucleus (Wang et al., 1993; Ingber, 1997). Force exerted via the ECM will have two effects:

(a) Release of mRNA and ribosomes attached to the cytoskeleton and translocation to the sites, where protein synthesis is required (Chicurel et al., 1998).
(b) Nuclear deformations or conformational changes of the chromatin that directly may affect the transcriptional activity (Bloom et al., 1996).

Accumulating evidence supporting this model in the regulation of cellular organization is largely based non-muscle cells (Chicurel et al., 1998; Lelievre et al., 1998; Thomas et al., 2002).

With respect to adaptation of skeletal muscle, the involvement of mechano-transduction in the regulation of adaptation of muscle size remains to be characterized. For lengthening of cardiomyocytes, it was shown that cytoskeleton desmin filaments transmit force onto the nucleus (Bloom et al., 1996). It was hypothesized that this could alter the conformation of the chromatin and possibly stimulate transcription. It should be realized that tensegrity effects have only been shown for relatively small cells (diameter in all directions $<20\,\mu m$). Since muscle fibers are very big multinucleated syncytia (for example adult humans: cross-fiber diameter $>50$ μm, 1 cm $<$ length $<30$ cm), it is very well conceivable that the muscle fiber consists of many units of tensegrity regulating muscle fiber size, i.e. the adaptations taking place within these local units.

In addition, changes in nuclear shape may also cause entry of [$Ca^{2+}$] into the myonucleus as was shown in isolated nuclei in response to osmo-mechanical stress (Itano et al., 2003).

However, direct mechanical signalling via the cytoskeletal molecule desmin was not confirmed by experiments with desmin knockout mice. After im-

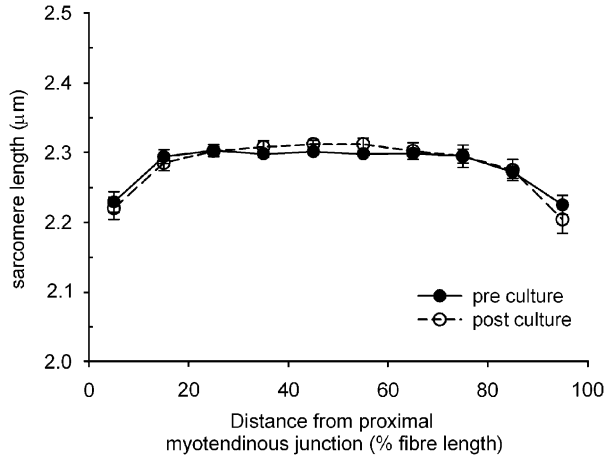


*Fig. 10*. Serial sarcomere length distribution in cultured single *Xenopus laevis* muscle fibers. Using laser diffraction techniques, saromere length was measured at intervals along the length of the muscle fiber-basal lamina-endomysium units cultured successfully for several weeks. Position along the fiber is quantified as deviation from the proximal myotendinous junction and normlized for muscle fiber length. Note the rather limited size and location of serial distribution of sarcomere length. Note also that these values are very much lower than those expected of the basis of finite-element modelling for fibers within a muscle exposed to epimuscular myofascial force transmission.

mobilization of mice muscles at high or low length, desmin knockout mice showed the same adaptations of the muscle physiological cross-sectional area, as well as the serial sarcomere number as for wild types (Shah et al., 2002). This may indicate that mechano-transduction via desmin is either not relevant in the regulation of muscle size, or, alternatively, that other cytoskeletal proteins replacing desmin or acting independently are involved in mechano-transduction as well.

## First attempts at a new approach: long-term culture of mature, single muscle fibers

Because of considerations of myofascial force transmission, an experimental setup for single muscle fibers from adult animals *in vitro* is required that allows long-term culture of muscle fibers with intact basal lamina and endomysium. In fact it should be feared that any method of isolation of muscle fibers that would damage the basal lamina–endomysium complex would lead to substantial changes of function of the muscle fiber and affect viability in a major way.

Using a culture setup as indicated, factors such as global or mean muscle fiber strain, hormonal composition of the culture medium as well as the type and degree of contractile activity can be manipulated independently during the culture. Such a system should allow investigation of the effects of the factors regulating adaptation of muscle fiber size independently, as well as allow study of their interactions.

A unique culture system for mature single *Xenopus* muscle fibers was developed by Lee-De Groot and Van der Laarse (1996), which meets most of these requirements. Using this system, these authors showed that it is feasible to culture mature single muscle fibers for 2 weeks. However, when using a serum-free culture medium, as culture time progressed twitch force declined and the muscle fibers were shown to be metabolically unstable (Lee-de Groot & Van der Laarse, 1996).

However, the feasibility of dissections of single muscle fibers with intact basal lamina and endomysium and their culture warranted further development of a system with the use of which, mature, single *Xenopus* muscle fibers could be cultured in stable conditions for up to months.

In close collaboration between the laboratories of Huijing and Van der Laarse, Jaspers developed a new culture medium and tuned the system such that muscle fibers can be maintained metabolically stable, while tetanic force as well as adaptation of the serial sarcomere number can be studied longitudinally. The success of this new, well-defined culture medium was proved, as we were able to culture single mature *X. laevis* muscle fibers for up to three months, while tetanic force was stable (Jaspers et al., 2001; Jaspers et al., 2004).

### Dissection and culturing of single *Xenopus* muscle fibers

Dissection of single *Xenopus* muscle fibers from small fascicles attached to remnants of tendon requires special skills. In *Xenopus* muscle this is generally somewhat easier than in mammalian muscle, because, in contrast to mammalian experimental animals, the dimensions of the muscle fibers (diameter up to 100 μm) are similar to those of muscle fibers of adult humans. Measurement of the relationship between muscle fiber cross-sectional area and the oxidative capacity of muscle fibers has shown that the metabolic fluxes of muscle fibers from *X. laevis* and humans are similar, whereas those of rodent muscle fibers are substantially higher (Van der Laarse et al., 1991). A drawback may be that the myonuclei of *Xenopus* are, in contrast with human muscle fibers, not located exclusively at the periphery of the fiber.

Single muscle fibers have been isolated aseptically from fascicles of the adult *X. laevis* iliofibularis muscle by cutting at the endomysia of surrounding muscle fibers and removing the muscle fibers surrounding a target fiber. Such dissection leaves the endomysium and basal lamina surrounding the target muscle fiber (see Fig. 9 of Jaspers et al., 2004).

In addition to the high control of the physiological conditions (overall strain, serum-free medium; for details see Jaspers et al., 2001, 2004), a very important methodological advantage is that the parameters of interest can be studied *longitudinally*, which eliminates the need for group statistics and avoids difficult decisions regarding control groups.

Using such methods, not only adaptation at the supra-molecular level but, using *in situ* hybridization, also the very early signs of preparation for adaptation can be studied(i.e. mRNA transcription) (Jaspers, 2002; Jaspers et al., 2002).

### Overall strain does not uniquely regulate muscle fiber size

Using a new culture medium, we investigated the effects of culture of the muscle fiber at a mean sarcomere length of 2.3 μm ($l_{2.3\,\mu m}$). At this length, the muscle fiber is just over its passive slack length. Before and after culture, the coefficient of variation in sarcomere lengths along the muscle fiber amounted to less than 2% (Fig. 10). Note that the value is very much lower than those obtained for muscle fibers during epimuscular myofascial force transmission as predicted by finite-element modelling (see "Finite-element models of epimuscular myofascial force transmission confirm relatively high serial and parallel distribution of muscle fiber strain"). During culture of up to 2 weeks at $l_{2.3\,\mu m}$, tetanic force was shown to remain constant and the serial sarcomere number was not changed (Jaspers et al., 2001). These data indicate similar rates of protein degradation and synthesis during culture at this fiber length.

In order to test whether overall fiber strain uniquely regulates $A_f$ and the serial sarcomere number, fibers were cultured (Jaspers et al., 2004) at positive overall strain (i.e. $\approx 5\%$ over $\ell_{2.3\ \mu m}$, referred to as "long fibers" below) or negative overall strain (i.e. $\approx 20\%$ below $\ell_{2.3\ \mu m}$, referred to as "short fibers" below). The culture period varied from 4 to 97 days.

Note that long fibers are cultured at sarcomere lengths over 2.4 μm, i.e. a length at which $Ca^{2+}$ influx from the ECM into the cytoplasm occurs (shown in frog fibers). For the long fibers, cultured up to 17 days, we did not detect any sign of hypertrophy as tetanic force remained stable or decreased slowly. Also, the serial sarcomere number showed no major effects of high strain. However, after 2 weeks of culture for two fibers, which were characterized by lower half-relaxation times than the others, a significant, but small, increase in the serial sarcomere number of approximately 4% was observed. For short fibers the serial sarcomere number remained unchanged, even after 97 days of culture. Surprisingly, tetanic force doubled over this period. However, acutely after the culture, the increase in tetanic force could be made to disappear immediately after the muscle fiber had been activated tetanically at a high length (i.e. over a mean sarcomere length of 2.3 μm) and tetanic force was again similar to its initial value before culture. This is a relatively ill understood phenomenon requiring further research.

It is concluded that culture muscle fibers at different overall strains neither induce atrophy or hypertrophy, nor a major change in the serial sarcomere number. Therefore, it is concluded that *in vitro* adaptation of $A_f$ and serial sarcomere number is not regulated by overall or local muscle fiber strain per *se*.

### Discrepancy between adaptation effects *in vivo* and *in vitro*

Comparison of such *in vitro* results with those of the classical experiments *in vivo* reveals strikingly different effects in response to long-term maintenance of muscle fibers at high or low lengths. Because of the isolation of the muscle fibers and the culture itself, the physical as well as the biochemical environment of the muscle fiber hasbeen altered compared with the *in vivo* situation. The discrepancy reported yields challenges to identify mechanisms that are active during adaptation *in vivo*, but which may not be active *in vitro* and vice versa.

#### *Molecular factors*

The presence of autocrine/paracrine growth factors at the sarcolemma, in combination of high overall strain, is usually required for induction of hypertrophy and an increase in serial sarcomere number. Since we are dealing with isolated muscle fibers, paracrine factors from neighboring muscle fibers are absent, but not from fibrocytes within the endomysium or micro-tendon.

It could be argued that autocrine growth factors may have been washed away by continuously pumping fresh culture medium through the culture chamber. However, if one would suppose that during culture growth factors were washed out, one would expect long muscle fibers to atrophy, and particularly short muscle fibers to atrophy and reduce their serial sarcomere number. This clearly did not occur.

As the basal lamina is expected to facilitate the presence of an unstirred layer near the sarcolemma of the already gel-like ECM, the direct flow of perfusate is expected to pass predominantly on the outside of the endomysial tunnel of the isolated muscle fiber. In addition, generally, the sulphated glycosaminoglycan branches of the giant proteoglycan molecules (Vlodavsky et al., 1987; Vukicevic et al., 1992; Mason, 1994; Tatsumi et al., 1998, as well as collagen IV

(Vukicevic et al., 1992) within the basal lamina bind several types of growth factors, i.e. the basal lamina acts as an anchor place for growth factors. Therefore, it also acts as a source of growth factors if concentrations fall and hence the basal lamina regulates the growth factor concentration. Therefore, a reduction in the concentration of endocrine growth factors, if any, in the neighborhood of their sarcolemmal receptors will not dramatic. There may be one notable exception: binding of IGF-1 to heparan sulfate was not shown (Vukicevic et al., 1992).

Therefore, it cannot be excluded that the concentration of IGF-1 could have been lowered, because of the absence of other binding proteins, other than most common constituents of the ECM (e.g. IGF-1 binding proteins; Florini et al., 1996, or serum proteins such as α1-acid glycoprotein and albumin; Lovich et al., 2001 and specific plasma globulins; Pardridge, 1981). It should be noted that albumin concentration increased within the ECM of mouse soleus muscle and rabbit tibialis muscle in response to immobilization (Wagatsuma & Yamada, 2000) and during low-frequency chronic stimulation (Heilig & Pette, 1988), respectively.

### *Mechanical factors*

The question that arises is whether isolation of a single muscle fiber for the culture does not remove the specific mechanical signals for adaptation of fiber size. In view of the very substantial effects of epimuscular myofascial force transmission, for example, on the serial distributions of sarcomere length within one muscle fiber (as indicated by the finite-element modelling), it is hypothesized that the removal of the tensile and shear effects of neighboring muscle fibers and neighboring muscle also modified or removed the mechanical signal for adaptation. If this is true, it means that the mechanical interactions, via neighboring muscle fibers and their endomysial–perimysial stromata, may potentially be major determinants of adaptation of muscle size. It should be noted that such interactions could originate from extra- or intramuscular sources, as close as other bundles within the same muscle, or as far away as antagonistic muscles. Not only the forces exerted by those sources, but particularly also their altered relative positions causing shear deformation of the material constituting their interfaces could potentially be important variables.

In view of the very substantial effects of epimuscular myofascial force transmission (for example on serial distributions of sarcomere length, as indicated by the finite-element modelling), it is hypothesized that the mechanical isolation of the muscle fiber decreased the signal for adaptation.

This could affect the muscle fiber in two ways. The removal of high local strains from the complex of cytoskeleton, basal lamina and endomysium of the muscle fiber would: (1) alter mechanical signalling at the myonuclei according to the paradigm of tensegrity (i.e. by changing cell shape or the shape of the tensegrity unit of the muscle fiber leading to for example expression of growth factors) and (2) alternatively, prevent exceeding the threshold for activation of signalling molecules at the sarcolemma leading to biochemical signalling within the muscle fiber to the nucleus and eventually enhanced expression of for instance growth factors.

When secreted into the ECM, such growth factors, in turn, will stimulate proteins synthesis over a more extended area along the length of the muscle fiber (autocrine effects) as well as that of its neighboring fibers (paracrine effects).

It may be somewhat surprising that, if mechanosignalling within the muscle fiber leads to the expression of growth factors, secretion to the ECM is still needed to activate biochemical-signalling pathways. In such reasoning, we hypothesize that the secretion of growth factors has as a major side effect to coordinate the adaptation of different units along the length of the muscle fiber and neighboring muscle cells. This would mean that effects of myofascially induced, variation of local tensile or shear strains along the muscle fiber as expected *in vivo* are averaged so that the degree of adaptation of a substantial number of presumed units of adaptation is similar, even though differences may be sustained over longer distances.

For example, the differentially increased MAPK activity within the fibers of rat medial gastrocnemius muscle according to location within the muscle (Csukly et al., 2002) may be related to such myofascial effects (see Huijing & Baan, 2001a) of remnants of the dissected neurovascular tract.

## Overall conclusions and discussion

In the present work, we reviewed results from experimental work as well as mathematical modelling for very different levels of muscular organization. In addition, we presented some new experimental results on epimuscular myofascial force transmission in *X. leavis* and on myofascial force transmission between antagonistic rat muscles to complete the picture presented.

This review demonstrates that, although insight in the mechanisms underlying adaptation of muscle fiber size is growing fast, our knowledge remains rather limited and often controversy reigns, because of a lack of control over the physiological conditions imposed by the experiments.

The lack of experimental control is implicit for *in vivo* work on humans, for which rather little control on experimental conditions of a target muscle is feasible (for example, on degree of activation, on values of locally exerted forces and local strains). On the other hand, one of the clear advantages of this type of work is its immediate applicability.

A little more control, but as we argue above for adaptation studies not a sufficient degree, is possible within animal experiments involving muscle bellies surrounded by their natural context of connective tissues or, more usually, in *maximally dissected muscle in situ*. It should be noted that in most adaptation studies using animal experiments (for example immobilization, surgical interventions) experimental conditions are imposed *in vivo*, while the adaptation effect is assessed for the fully dissected condition of the *in situ* muscle. Therefore, we argue that adaptation effects should also be assessed with myofascial effects active. If this is not done, potential effects of epimuscular myofascial force transmission will be neglected. A clear example can be derived from observations in patients suffering from spastic paresis of the flexor carpi ulnaris muscle (FCU). This affliction leads to the typical palmar flexed and ulnar abduction position of the wrist. After dissection of the muscle for approximately 50% along the length of the muscle belly (necessary to allow transfer of the FCU tendon insertion to the extensor side subsequent to the experiment), measurements of length–force characteristics yielded no indication for the reason of this contracture (Smeulders et al., 2004b): neither high passive forces, nor unusually high muscle lengths were encountered at muscle–tendon complex lengths corresponding to the maximal dorsiflexion position possible. This leads to the hypothesis that the contracture accompanying the spastic paresis was caused by myofascial interaction of the FCU with neighboring muscles or other tissues.

From this example, it is also clear that we need to pay much more attention to potential adaptation of the connective tissues within and surrounding the muscle and its acute and long-term effects on muscular properties.

The discrepancy between *in vivo* and *in vitro* data on adaptation presented in paragraphs above (e.g. effects on muscle fiber diameter and serial sarcomere number in whole muscle kept at low lengths, but not in isolated muscle fibers cultured under such conditions) demonstrates the value of contrasting approaches at the extremes of the muscular organization (i.e. single fiber *in vitro* vs whole muscle in its natural surroundings).

We argue here that we do need all levels of experimentation presented, but that a better integration of results and developed knowledge is necessary. In addition, a better tuning of the experiments to the specific goals attainable at the specific level of organization is necessary (e.g. major mechanisms are not likely to be discovered in an *in vivo* experiment). We also suggest that uncovering knowledge of the mechanisms underlying adaptation of muscular size requires much better control of experimental conditions, as obtained for example through *in vitro* experiments on mature muscle fibers. Sophisticated techniques must be developed in order to study the effects of mechanical signals that are applied to the muscle fiber at the basal lamina and sarcolemma or directly on the myonuclei and how such signals trigger adaptation of muscle fiber size directly within the nuclei and/or by interactions with biochemical factors at the sarcolemma.

In spite of having gained knowledge of fundamental mechanisms of adaptation, the work has only started, because we need to consider the question how these mechanisms are affected and how their effects are modulated by activity at higher levels of muscular organization. For example, how do the distributions of sarcomere lengths likely to be present within a muscle fiber that is surrounded by adjacent muscle fibers of the same muscle or by nearby fibers of other muscles and extramuscular connective tissues affect the processes of adaptation?

Therefore, knowledge on fundamental adaptation mechanisms should be tested for *recognition of effects* at the more complex levels of organization. Potential modification of its effects at these higher levels of muscular and connective tissue organization should be quantified. In that way, integration will become possible if results are obtained at all levels of experimentation.

There are other types of important interactions between the studies of the different levels of organization. For example, once aware of the presence of distributions of serial sarcomere lengths, researchers working at the level of the isolated muscle fiber may try to impose such distributions on their isolated cells. In other words, there should be two-way traffic of information and ideas in people studying the different levels of muscular organization.

It is clear that non-invasive imaging in human subjects or patients will need to play a very important role in the process of recognition of effects at the highest level of organization of tissues. Presently, two types of techniques are available for that: ultrasound imaging and MRI. The former has the advantage of being much cheaper and more easily applied than the latter. However, the question whether ultrasound imaging has sufficient contrast and resolution to play a major role at the process of recognition of effects of more fundamental mechanisms at the *in vivo* level should be raised. MRI also has its limitations regarding contrast, but application of advanced radio-frequency techniques to give the

tissue magnetic properties that can be recognized within the image may help overcome this problem.

It is very unlikely that much mechanism will be uncovered in studies performed at the *in vivo* level. Epimuscular myofascial force transmission is a good example in this case: *in vivo* imaging had been around for years, without leading to any discovery of myofascial effects, because measurement of locally exerted muscle forces was essential to its recognition and proof. The *in vivo* shifts of muscles (or their parts) with respect to each other and with respect to neighboring other tissues can be quantified quite well, even using ultrasound imaging (e.g. Bojsen-Møller et al., 2004). However, the interpretation of effects of such relative movements and changes of relative position remains very hard, unless information regarding effects of myofascial movement, obtained from better-controlled animal experiments, is integrated. We also suggest that imaging and analysis of muscles in planes of view that are quite unusual today (i.e. imaging planes other than only the mid-longitudinal view of the muscle showing recognizable fascicle patterns) may be quite necessary to recognize the mechanism of findings– based on animal experimentation.

Finite-element modelling may help to study features at any level of organization that are inaccessible to direct experimentation and can be used also to feed and support the theoretical framework within which results are to be interpreted.

If successful, the suggested integrative multilevel approach to the study of muscular adaptation is expected to lead also more directly toward applications that will allow manipulation of adaptive processes for the use in clinical interventions, as well as training-related goals. Considering the wide range of studies and disciplines needed for such study of adaptation, close collaboration of many different types of laboratories is needed. The departments and faculties to which those laboratories belong should also train their students to study at the interdisciplinary level to such a degree that communication across discipline boundaries is possible and the sometimes present abyss separating the fundamental and applied scientists is bridged.

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